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FATTY ACID PATTERNS IN ANAEROBIC AND FACULTATIVELY
ANAEROBIC 'CORRODING BACILLI'

by



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A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES
IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE
OF MASTER OF SCIENCE

DEPARTMENT OF BACTERIOLOGY

EDMONTON, ALBERTA

FALL, 1970

ABSTRACT

Fatty acids of 12 strains of Gram-negative, anaerobic and facultatively anaerobic 'corroding bacilli' classified as Bacteroides corrodens on the basis of morphological, cultural, and biochemical characteristics were extracted from the cells and analyzed as methyl esters by gas-liquid chromatography. Visual and quantitative comparisons of the fatty acid spectra allowed rapid differentiation of the facultatively anaerobic strains from the anaerobic strains. The latter were further subdivided into two groups. Each group possessed its characteristic fatty acid methyl ester profile typical of all strains within the group. The facultative strains B. corrodens NCTC 10596 (333/54-55), B. corrodens NCTC A40/68, B-916, S-209, 53P and 53W were characterized by large percentages of palmitic and octadecenoic acids with smaller percentages of palmitoleic acid and small amounts of lauric, myristic and stearic acids. The anaerobic strains, B-912, EDMH-1, 4282 (Lane) and 4053 contained a large percentage of octadecenoic acid and smaller amounts of palmitic, lauric, myristic and stearic acids. The anaerobic strains 3936 and 4482 produced an unidentified acid which represented as much as 93% of the total fatty acids. This fatty acid profile was shown to be similar to that of the Bacteroides species included in this study, namely B. fragilis, B. oralis and B. melaninogenicus.

Analysis of metabolic end-products produced by these organisms proved to be only an aid in the classification of these bacteria. Its usefulness can be seen in the demonstration of the heterogeneity of the Bacteroides group. The results strengthen previous suggestions that the agar-corroding strains be reclassified.

Eight strains of Pasteurella multocida representing different serotypes, together with Pasteurella pseudotuberculosis, Pasteurella haemolytica and Haemophilus aphrophilus were included as examples of genotypically different Gram-negative bacilli. H. aphrophilus NCTC 5886, once suggested as being related to the corroding strains, exhibited a fatty acid profile similar to that of the Pasteurella strains. These were characterized by large amounts of palmitic and palmitoleic acids in almost equal proportions and a smaller amount of myristic acid.

Indications are that fatty acid patterns can be useful for the differentiation of closely related organisms, but in some instances, biotypes distinguishable by other tests may have virtually identical fatty acid profiles.

ACKNOWLEDGEMENTS

I wish to express my appreciation to Dr. F. L. Jackson, Professor and Chairman, Department of Bacteriology, and also to Dr. R.L.S. Whitehouse, for advice and guidance in the preparation of this thesis.

I would also like to express thanks to Mr. L. A. Howard for assistance with the gas chromatograph and to Mrs. E. Davies for typing the thesis.

LIST OF ABBREVIATIONS AND DEFINITIONS

GLC	- gas-liquid chromatography
P-GLC	- pyrolysis-gas-liquid chromatography
PTGC	- programmed temperature gas chromatography
ECL	- equivalent chain length
i-	- iso; refers to methyl group on the penultimate carbon
a-	- anteiso; refers to methyl group on ante-penultimate carbon
n-	- normal; refers to a saturated, straight chain fatty acid
Fig.	- Figure
w/v	- weight per volume
v/v	- volume per volume
rpm	- revolutions per minute
UAH	- University of Alberta Hospital
G+C content	- refers to the DNA base ratio: $\frac{G+C}{G+C+A+T}$

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I

INTRODUCTION AND LITERATURE REVIEW

INTRODUCTION

Classification of bacteria at the present is based primarily on morphological, cultural, biochemical and serological characteristics. The difficulties in assigning certain bacterial strains to their taxonomic position by commonly used criteria has stimulated a search for additional characteristics. This has led to the consideration of chemical composition as an added criterion for establishing taxonomic relationships among microorganisms. One of the initial attempts at utilizing chemical differences for the identification of microorganisms was published in 1956 by Mattick and co-workers (43), who reported that it was possible to use paper partition chromatography in the differentiation of microorganisms. Development of this concept has led bacterial taxonomists to look at several selected compounds such as proteins, amino acids, carbohydrates, lipids, etc. as supplementary criteria for organisms of uncertain taxonomical status. It seems reasonable that similarities as well as differences in cellular chemical composition could provide a good, if not better, theoretical basis for classification than what is presently used because one can relate differences in chemical composition to natural or evolutionary relationships, provided the bacteria are grown under defined conditions.

Characterization of cellular components often requires complex, selective and time-consuming techniques. However, recent

development and refinements of the technique of gas-liquid chromatography has encouraged the use of this approach because small samples may be analyzed rapidly and accurately. Gas-liquid chromatographic techniques permit the determination of complex mixtures of volatile products or their volatile derivatives within a few minutes following the injection of a few microliters of sample. For example, fatty acids ranging from 10 to 20 carbon atoms in length may be determined by chromatographing their methyl esters following removal of the acids from bacterial cells by direct saponification procedures. Compounds present in nanogram and picogram quantities can be efficiently detected and measured by the use of appropriate detectors.

Several gas-liquid chromatographic approaches are being used for rapid characterization of microorganisms. These include analysis of pyrolysis products of whole bacterial cells, analysis of specific chemical compounds extracted directly from the cells as well as analysis of media extracts or of head-space gas for metabolic products synthesized by microorganisms during their growth.

Many metabolic products produced by microorganisms are volatile and thus may be analyzed by gas chromatography without conversion to volatile derivatives, which is proving to be a major advance in the more sensitive, more accurate and more rapid determination of some biochemical characteristics such as fermentation patterns. The presence of low levels of synthesized alcohols, amines and acids can be determined either qualitatively

or quantitatively. Previous methods for the determination of fermentation products are generally not as sensitive and sometimes difficult to interpret.

The classification of some Gram-negative, non-spore-forming, agar-pitting, anaerobic and facultative rods has proved to be difficult due to the fact that these organisms are fastidious in their growth requirements and that negative results are usually obtained in many of the cultural and biochemical tests routinely used. This investigation was undertaken with two objectives in mind: (i) to determine whether unidentified isolates tentatively designated as belonging to the family Bacteroidaceae could be placed into a recognized genus by similarity in fatty acid composition and fermentation patterns and (ii) to determine whether strains of so-called B. corrodens could be identified or separated from one another by means of their fatty acid spectra and metabolites produced in the culture medium. Ten strains of biochemically well-characterized Gram-negative bacilli were used for comparison purposes.

LITERATURE REVIEW

The technique of gas-liquid chromatography was first introduced by James and Martin in 1952 (28) but was not proposed for taxonomic differentiation of microorganisms until 1963 when Oyama (55) suggested a technique involving a chromatographic characterization of the pyrolysis products of bacteria or substances of biological origin to determine the possible existence of life in extraterrestrial environments.

In the same year Abel et al. (1) made the first attempt at using GLC to correlate chemical composition of bacteria with taxonomic classification. They were able to differentiate between the families of Enterobacteriaceae, Bacillaceae, Micrococcaceae, and Parvobacteriaceae by comparing the chromatograms of the fatty acid methyl esters of the lipids extracted from selected representatives of each of these families. Differentiation at the genus and species level was reported as difficult.

In a similar study, Brown and Cosenya (9) in 1964 compared the fatty acid spectra of three spherical bacteria which differed in their cellular arrangement, Gram-stain reaction and habitat. They distinguished between the genera Gaffkya and Micrococcus of the family Micrococcaceae. In the same year, Yamakawa and Ueta (75) were able to differentiate at the species level by GLC analysis of cellular fatty acids and carbohydrates of Neisseria meningitidis Groups A, B, C, D, N. flavescens, N. perflava,

N. subflava, N. haemolysans, and N. gonorrhoeae. Lauric, myristic palmitic, hexadecenoic, octadecenoic, β -hydroxylauric and β -hydroxymyristic acids and galactose, glucose, glyceromannoheptose were the common constituents of these bacteria except N. haemolysans, which differed from other Neisseria in both fatty acids and sugar composition.

Reiner (57) in 1965, was one of the first to attempt the differentiation of related bacteria by analysis of their pyrolysis products by GLC. He detected chemical differences between bacterial strains of similar antigenic or pathogenic character by examining the 'pyrogram' unique to each strain. Although the organisms showed similar profiles, they differed from each other in their peak ratios.

Also in 1965, Bassette and Claydon (5) outlined a simple method for the rapid characterization of bacteria. With the aid of GLC, they analyzed head-space vapors produced by bacteria growing in milk. Pure cultures of organisms with widely different morphological and biochemical characteristics gave markedly different chromatographic patterns whereas closely related organisms gave similar profiles but sufficient differences existed to distinguish them from each other. The organisms used in this study were several species of Lactobacilli, Streptococci and Aerobacter aerogenes, Escherichia coli, and Achromobacter lipolyticum. Using this technique, Bawden and Bassette (6) differentiated between several isolates of E. coli and A. aerogenes on the basis of two unidentified

compounds present in relatively large quantities in A. aerogenes and in trace amounts only in E. coli.

As a result of these first publications, GLC began to contribute a mass of information towards the clarification of taxonomic relationships among microorganisms. The major contributions have come from the analysis of cells and growth medium for fatty acids. The study of bacterial lipids began in 1920 with the work of R. J. Anderson (2) who isolated and characterized the lipids of the tubercle bacillus. Since then, the accumulation of knowledge concerning lipid composition of bacteria by various techniques such as fractional distillation, crystallization, countercurrent distribution and partition and absorption chromatography has been increasing steadily.

The total lipid content in bacteria ranges from 1 to 10% of the dry cell weight (53). Gram-positive organisms are known to contain greater amounts of lipids than Gram-negative organisms. These are distributed in cytoplasmic inclusions, cell walls and protoplasmic membranes. Lipid globules are found in several bacterial species such as Azotobacter, Rhizobium, Spirillum, Bacillus and Corynebacterium diphtheriae (53). Cell walls of Gram-positive organisms contain 1 to 3% lipid, whereas Gram-negative organisms contain 10 to 20%; some organisms have as much as 26% lipid (33). Cytoplasmic membranes contain between 10 and 30% lipid (53).

The chemical composition of bacterial lipids has been

reviewed by O'Leary (53) who first, grouped them according to their chemical natures, and then in the chemical combinations in which they occur.

Saturated straight chain acids (normal acids) of less than 12 carbon atoms are detected in small amounts in all bacterial species studied so far. Higher, even-numbered, saturated, straight chain fatty acids constitute a larger proportion of the total fatty acid content (C_{12} to C_{28}), palmitic acid (C_{16}) occurring most frequently and in larger amounts than the others. C_{20} to C_{28} acids are not encountered in all species. C_{28} or longer have only been reported in mycobacteria.

Hydroxy acids have been detected in several species of bacteria such as Bacillus, Pseudomonas, Micrococcus and Azotobacter. The so-called lipid granules in bacteria are made up of poly β -hydroxybutyric acid which can account for as much as 50% of the dry weight of a bacterial cell (64). Corynebacterium diphtheriae is known to contain several complex hydroxy acids.

Branched chain acids have been reported in several species such as Bacillus, Sarcina and Corynebacteria, the latter containing several complex branched chain acids.

Cyclopropane acids have been found in several bacteria including species of Lactobacillus, Escherichia coli, Clostridium butyricum, Bacillus subtilis and Pasteurella pestis.

Unsaturated fatty acids constitute a major portion of the fatty acids of all bacteria; unsaturated fatty acids of 16 to 18

carbon atoms are most commonly found. Unsaturated acids greater than C_{18} have been reported only in Corynebacteria.

Bacterial lipids are found as free fatty acids or in combination with other cellular components. As much as 20% of the total fatty acids can occur free in some bacteria such as Corynebacterium diphtheriae, Bacillus megatherium and Salmonella typhimurium. Fatty acid polymers, glycerides, waxes, phospholipids, glycolipids, lipopeptides and lipoproteins and bound lipids are encountered in bacteria. Glycerides or neutral lipids are found in low concentration or not at all in bacteria which is contrary to plant and animal cells. Waxes appear limited to Mycobacteria and Corynebacteria. Phospholipids frequently comprise major portions of the total lipid content. Of the total lipids of Bacteroides melaninogenicus 97% are phospholipids (63). The term 'bound lipids' refers to the lipid constituents of the bacterial cell which are firmly combined with carbohydrates and/or proteins and can be extracted only with organic solvents following liberation by some hydrolytic procedure.

In a review article, Kates (33) attempted to correlate fatty acid composition with taxonomic classification on the basis of the Gram-stain reaction. Gram-negative organisms have high proportions of normal, even-numbered, saturated and unsaturated acids and odd-numbered cyclic acids; while Gram-positive organisms have high proportions of saturated odd-numbered branched chain acids and relatively low amounts of straight chain saturated or unsaturated

acids. However, there are exceptions. Among the Gram-positive organisms which did not fit into this generalization were the Lactobacteriaceae family and some of the Clostridia (e.g. Clostridium butyricum) which have fatty acid spectra resembling those of Gram-negative bacteria. Among the Gram-negative bacteria, members of the Azotobacteriaceae family do not have cyclopropane acids which are characteristic of Gram-negative bacteria. The Corynebacteria, Gram-positive organisms, seemed a group apart in that they do not contain appreciable amounts of simple branched chain acids but have C_{20} to C_{28} saturated and unsaturated acids and complex hydroxy branched C_{32} acids.

In the same review article Kates found better correlation with taxonomic classification when comparing fatty acid composition at the family level than with the Gram stain. Table I adopted from this publication, shows the principle fatty acids of some bacterial families. As Kates points out, lipid composition is distinguishable at different levels of bacterial classification. The Eubacteriales appear to be a less complex order than the Actinomycetales except for the Corynebacteriaceae family, which has complex lipid patterns. On the family level, distinguishable lipid patterns are evident although there are exceptions.

With the availability of GLC, fatty acid composition as a taxonomic criterion has received increased emphasis. By examination of the fatty acid spectra for a group of microorganisms, differentiation is frequently made at the genus level and in some cases at

TABLE I

SUMMARY OF DATA ON LIPID COMPOSITION OF BACTERIA
ACCORDING TO THEIR TAXONOMIC CLASSIFICATION

Bacterial Family	Major Fatty Acids ^a
Eubacteriales (Gram-negative)	
Enterobacteriaceae	16:0, 16:1, 18:1, 17:cy, 19:cy
Azotobacteriaceae	16:0, 16:1, 18:1
Rhizobiaceae	16:0, 18:1, 17:cy, 19:cy
Pseudomonadales	
Pseudomonadaceae	16:0, 16:1, 18:0, 18:1, 19:?
Eubacteriales (Gram-positive)	
Lactobacillaceae	16:0, 16:1, 18:1, 19:cy
Bacillaceae	
Bacilli	13:br, 15:br, 17:br
Clostridia	16:0, 16:1, 18:1, 17:cy, 19:cy
Micrococcaceae	15:br, 17:br
Corynebacteriaceae	16:0, 16:1, 18:0, higher complex acids
Actinomycetales	
Mycobacteriaceae	16:0, 16:1, 18:0, 18:1, 19:br, mycolic acids

a Abbreviations: The first number indicates chain length, the second the number of double bonds; cy signifies the cyclopropane ring; br signifies a branched chain.

(Kates, M. 1964. Adv. in Lipid Res., 2: 17 - 90.)

the species level. Although GLC methods alone cannot always be used for the identification of these components, tentative identifications can be made, or a simple visual comparison of fatty acid profiles can be used as a basis for taxonomic differentiation of bacteria.

Kaneda (30) has clearly elucidated the taxonomic relationships of several species of the genus Bacillus. Fatty acids produced by 22 strains of 10 species of Bacillus, namely B. alvei, B. brevis, B. cereus, B. circulans, B. licheniformis, B. macerans, B. megatherium, B. polymyxa, B. pumilis, and B. subtilis were characterized by GLC retention volumes on two columns, adipate and Apiezon L. Further characterization was carried out by bromination and hydrogenation procedures. All 10 species were shown to produce 6 branched chain (C_{14} to C_{17} iso and anteiso) and 2 normal ($n-C_{14}$ and $n-C_{16}$) fatty acids as major fatty acid constituents. However, one species, B. cereus, in addition to the above, produced four extra fatty acids, three branched chain ($a-C_{13}$, $i-C_{12}$ and $i-C_{13}$) and one unsaturated fatty acid ($n-C_{16:1}$). Furthermore, B. cereus produced the fatty acid ($i-C_{15}$) in greater quantity than any other species on the medium used.

In another study, Kaneda (31) compared the fatty acid composition of two other species of Bacillus which are taxonomically very close to B. cereus, namely B. thuringiensis and B. anthracis. Both produced branched chain fatty acids as major constituents of the total fatty acids (70 to 80%), which agrees with the 10 species

previously discussed. Like B. cereus, both species produced some fatty acids with less than 14 carbon atoms and $i\text{-C}_{15}$ in significant proportions. Unlike B. cereus both species produced an additional normal ($n\text{-C}_{15}$) and 3 additional monounsaturated ($i\text{-C}_{16:1}$, $i\text{-C}_{17:1}$ and $a\text{-C}_{17:1}$) fatty acids. The overall fatty acid composition for these two species consisted of 16 major fatty acids: 9 branched ($i\text{-C}_{12}$ to $i\text{-C}_{17}$, $a\text{-C}_{13}$, $a\text{-C}_{15}$ and $a\text{-C}_{17}$), 3 normal ($n\text{-C}_{14}$ to $n\text{-C}_{16}$) and 4 monounsaturated ($i\text{-C}_{16:1}$, $i\text{-C}_{17:1}$, $a\text{-C}_{17:1}$ and $n\text{-C}_{16:1}$) in addition to some minor fatty acids. Quantitative differences existed between these two species. B. thuringiensis produced higher proportions of $i\text{-C}_{13}$, $a\text{-C}_{13}$ and $i\text{-C}_{14}$ fatty acids than B. anthracis. In this respect, B. thuringiensis resembles B. cereus. Thus quantitative as well as qualitative differences may be used as a criterion for species differentiation.

In a previous publication, Kaneda (29) studied the factors affecting the relative abundance and the biosynthesis of branched chain fatty acids in B. subtilis. He suggests that the existence and proportions of chain branching may be significant as indicating different metabolic mechanisms, hence possibly of importance in identification and taxonomy of bacterial species.

Moss et al. (49) have investigated in detail 27 isolates of Corynebacterium acnes by a comparison of their cultural characteristics and GLC determination of fatty acid composition. This organism forms part of an ill-defined group of Gram-positive, anaerobic bacteria frequently called 'anaerobic diphtheroids'. The American

Society for Microbiology's subcommittee on Lactobacillaceae has recommended that several genera from this family, namely Eubacterium, Catenabacterium, Ramibacterium and Cillobacterium be placed in the Propionibacteriaceae family and that anaerobic species of Corynebacterium be placed in the genus Propionibacterium. Basing their investigation on this proposal, Moss et al. included representative species of the genus Propionibacterium, namely P. acnes, P. shermanii and P. freudenreichii in their study. All strains of C. acnes and P. acnes showed uniformity in their biochemical characteristics; P. freudenreichii and P. shermanii differed slightly; however, cell cultures produced similar fatty acid spectra which were characterized by a large amount of a C₁₅ branched chain acid (24 to 49% depending on the strain).

In another study Moss and Cherry (47) demonstrated that P. acnes could be differentiated from P. freudenreichii and P. shermanii on the basis of the point of branching of the methyl group in the C₁₅ branched chain fatty acid, that is, i-C₁₅ being most abundant in P. acnes and a-C₁₅ being the major fatty acid in the other two species. On the basis of this finding Moss et al. (48) further investigated 40 strains representing 7 species of Propionibacterium and 9 cultures of anaerobic corynebacteria. They were unable to distinguish between two groups of Propionibacterium, that is (i) P. freudenreichii and P. shermanii containing a-C₁₅ as the major acid, and (ii) 5 other species namely P. arabinosum, P. jensenii, P. pentosaceum, P. thoenii and P. zeae which contained i-C₁₅ isomer as its major fatty acid.

The corynebacteria resembled the second group. Thus fatty acid composition seems to support the suggestion that C. acnes be reclassified in the genus Propionibacterium. Hence the point of branching of a fatty acid may make one species different enough to differentiate it from another.

With the aid of GLC, Moss and Lewis (50) have characterized 41 strains representing 13 species of Clostridium. They also compared the cellular fatty acid composition of the toxigenic types and of food-poisoning strains of C. perfringens. By visual and quantitative comparisons of the fatty acid spectra they were able to distinguish C. perfringens, C. sporogenes, and C. bifermentans from each other and from the other 10 species studied. C. perfringens strains were distinguished by their high percentage of lauric (C_{12}) and myristic acids (C_{14}). There were quantitative differences between these two acids among the strains studied but no major differences in the fatty acid patterns were noted among the different toxigenic types nor among the food poisoning strains. C. sporogenes strains were grouped on the basis of an unidentified peak which varied in concentration among the strains studied but was unique to this group. C. bifermentans strains had no unique or major distinguishing peaks. The other 10 species were grouped together on the basis of the large percentage of myristic, palmitic and stearic acids, however quantitative differences did exist. No attempt to further characterize this group was made. Thus successful differentiation among closely related species and among certain

strains of bacteria was accomplished. The presence of an unidentified compound unique for a particular group may prove useful in more precise identification of a particular organism.

Ifkovits and Ragheb (25) attempted to differentiate among various rumen bacteria by GLC determination of their fatty acid composition: 21 pure cultures of rumen bacteria representing 12 genera and 14 species were included in this investigation. They were not able to differentiate between families or genera due to overlapping similarities and differences in fatty acid composition. At the species level, however, differentiation was possible on the basis of the major peaks present. Quantitative differences were observed among strains of some of the organisms. They conclude that on the basis of cellular fatty acid composition of rumen bacteria, classification is only possible at the species level. They do not agree with Abel et al. (1) that classification is possible at the family and genus level, saying that their findings were based on limited numbers of organisms examined for each family.

Lewis et al. (40) analyzed by GLC 9 species of Neisseria for cellular fatty acids: N. sicca, N. mucosa, N. flava, N. flavescens, N. perflava, N. subflava, N. catarrhalis and several serotypes of N. meningitidis. In addition to the fatty acids generally found in bacteria, they found an unidentified compound which was eluted from a nonpolar column between methyl laurate and methyl myristate. This component has apparently not been found in other bacteria, and

in the Neisseria group it was found only in N. catarrhalis.

Previous publications (10, 36) have questioned the position of this organism in the genus Neisseria.

Modak et al. (45) have studied the lipid composition of three strains of Salmonella with the aid of GLC. S. typhi, S. paratyphi A and S. paratyphi B exhibited similar spectra of fatty acids ranging from C_{12} to C_{22} , C_{16} (palmitic) constituting more than 60% of the total fatty acids. Quantitative differences were observed. In this study the organisms were cultured in both nutrient broth and a defined medium. Quantitative as well as qualitative differences were observed. On the basis of this observation, Modak et al. suggest that the loss of virulence in pathogenic species when transferred from natural to defined conditions might be a result of fatty acid alterations since it is now known that the infective unit or endotoxin of many bacterial species has lipid as one of its constituents.

Other GLC publications have appeared in which alterations in lipid composition have been correlated with bacterial variation. Brian and Gardner (7) in a comparison of the fatty acid composition of Vibrio cholerae and its rugose variants, have detected large quantities of C_{17} and C_{19} cyclopropane acids in the variants whereas they were undetected in the parent cultures. They suggest that the presence of considerable amounts of cyclopropane fatty acids in the variants may be related to their ability to grow in adverse environmental conditions whereas the parent strains will not.

Nesbitt and Lennarz (51) have studied the lipid composition of Proteus P₁₈ in both the bacillary and L form. They demonstrated that this morphological change was accompanied by an increase in the percentage of extractable lipids. The fatty acid composition of the extractable lipids was qualitatively identical in both bacterial forms. The difference between the two forms was characterized by a four-fold increase in C₁₆ unsaturated fatty acid and a three-fold decrease in C₁₇ and C₁₉ cyclopropane fatty acids in the L-form. The L-form also contained an unidentified component representing 7.2% of the total extractable lipids which was not detected in the bacillary form.

Weinbaum and Panos (73) have correlated the marked decrease in cyclopropane acids in the filamentous forms of Escherichia coli with the morphological change in the cell surface.

The fatty acid composition of Staphylococcus aureus strains differing in antibiotic sensitivity was compared by Redai et al. (56). Although the sensitive and resistant strains exhibited similar profiles, quantitative differences were observed. The resistant strains showed greater percentages of C₁₇ and C₁₉ cyclopropane or branched chain fatty acids.

Karkas et al. (32) have carried out a study on the fatty acids of mutants and variants of Escherichia coli. The wild type strains B, K 12 and 15 differed distinctly in their fatty acid patterns. Among the mutants examined, the thymine auxotrophs and streptomycin-resistant mutants exhibited fatty acid patterns

similar to those of the respective parent wild types. However, all methionine auxotrophs examined showed fatty acid profiles that differed quite markedly from those of the parent strains in both the complete medium and the minimal medium supplemented with sufficient methionine for normal growth; in the latter case the differences were even more obvious. Karkas et al. postulate that one or more pleiotrophic genes are involved and that even though an auxotroph is returned to normalcy when given its required growth substance, it still remains quite different from the wild type parent.

To better evaluate the data available on the fatty acid composition of bacteria, it is of prime importance that the nature of the medium and the conditions under which the culture is grown be known. The amount and composition of lipids in bacteria have long been known to vary with the culture medium, the age of the culture and the temperature in which they are cultured.

Among the various nutritional factors which have been reported as influencing the fatty acid content of bacteria are the amounts and relative proportions of acetate, carbohydrate, lipid and nitrogenous substances present in the medium.

Dunlap and Perry (17) described the fatty acid composition of three hydrocarbon-utilizing bacteria (Brevibacterium sp., Mycobacterium sp., and Nocardia sp.) cultured on various substrates. The major fatty acids in acetate-grown cells were: $C_{16:0}$, $C_{16:1}$, $C_{18:1}$ and branched $C_{19:0}$; in propane-grown cells: $C_{15:0}$, $C_{17:0}$,

C_{17:1}, C_{18:1} and branched C_{18:0}. When the Mycobacterium sp. was grown on n-alkanes (13 to 17 carbons in length), the major fatty acid in the cells was of the same chain length as the substrate.

Marr and Ingraham (42) demonstrated the effect of nitrogen or carbon limitation in the culture medium on Escherichia coli. A drastic increase in the proportion of saturated fatty acids (principally palmitic acid) at the expense of the unsaturated fatty acids (hexadecenoic and octadecenoic) was observed when the cells were grown with ammonium as the limiting nutrient. Cells harvested from the glucose-limited culture showed only a slightly higher content of unsaturated fatty acids than cells from the corresponding batch culture.

Abel et al. (1) noticed some changes in the fatty acid distribution of Escherichia freundii and Aerobacter cloacae when grown in trypticase soy broth and Koser citrate medium. They state that the changes were of sufficient magnitude to make species identification difficult but the pattern characteristics remained distinctive of the family.

Moss and Lewis (50) compared the fatty acid composition of two Clostridium perfringens strains in tryptose phosphate broth and peptone broth. They demonstrated that growth medium influenced the relative amounts of some of the principal acids (a difference of 11% in lauric acid and 12% in arachidic acid) but even with these differences grouping of these strains was not limited since the major distinguishing fatty acids of C. perfringens (lauric and

myristic acids) were present in relatively large amounts in cells grown in either medium.

Among the environmental factors which have been reported to influence the fatty acid composition of bacteria are the oxygen supply, pH, temperature and age of the culture when harvested.

Váczí et al. (70) have studied the effect of oxygenation and pH on Staphylococcus aureus. A decrease in the pH of the culture medium was accompanied by an increase in the amount of unsaturated fatty acids. Similarly, an increase in oxygenation (shake vs static culture) produced an increase in the amount of unsaturated fatty acids. Kaneda (30) reported similar results in his studies on the genus Bacillus. Cells grown in submerged cultures had a higher proportion of unsaturated fatty acids than cells grown on a solid medium due to the fact that the rate of diffusion of oxygen in a solid medium is probably slower than in a liquid medium. Knivett and Cullen (37) demonstrated an increase in cyclopropane fatty acids in Escherichia coli grown at low pH.

Marr and Ingraham (42) demonstrated the effect of temperature on the composition of fatty acids in E. coli grown in glucose minimal medium at 10, 15, 20, 25, 30, 35, 40 and 43°C. and harvested during exponential phase. As growth temperature decreased, the proportion of unsaturated acids increased and the proportion of cyclopropane acids decreased. With increasing temperature, the most abundant saturated fatty acid (C_{16}) increased. They also demonstrated that cells harvested in the stationary phase contained larger amounts of

cyclopropane fatty acids than cells harvested during exponential growth.

From these publications it is obvious that a detailed knowledge of the medium and culture conditions is essential for any valid interpretation of data regarding classification of bacteria on the basis of cellular fatty acids. Although it has been demonstrated that the overall spectrum is not always qualitatively changed, the relative proportions of each component has been shown to be a deciding factor in species and even strain differentiation.

The application of GLC procedures to the analysis of metabolic products of bacteria is also proving to be a major advance in a more rapid and accurate characterization of microorganisms. Several bacteria have been differentiated by their fermentation products; for example, species of Lactobacillus produce primarily lactic acid. Other genera or families of bacteria have been distinguished by the production of propionic, acetic and butyric acid as well as ethanol. This approach to the rapid detection or differentiation of microorganisms is based on the fact that volatile products or other products which can be converted into volatile derivatives can be found by the use of highly sensitive detectors. The three types of detectors commonly used are, in increasing order of sensitivity: thermal conductivity, flame ionization, and electron capture detectors.

Mitruka and Alexander (44) demonstrated that concentrations in the nanogram (10^{-9} g) and occasionally in the picogram (10^{-12} g)

range would yield a significant response with the flame ionization and electron capture detectors. They postulated that metabolites elaborated by the equivalent of less than a single cell of Bacillus cereus, Streptococcus faecalis or Proteus vulgaris were sensed by the electron capture detector.

Another advantage of GLC is its rapidity in the analysis of complex mixtures of volatile products which cannot always be separated by conventional techniques. Turner and Gilmour (69) have analyzed the fermentation products of Clostridium butyricum using both GLC and standard analytical procedures. Their report confirms the greater speed and simplicity of GLC.

Moore et al. (46) have examined the fermentation pattern of 20 Clostridium species using both GLC and column chromatography. They were able to divide these cultures into eight distinct groups on the basis of characteristic fermentation patterns. It was sometimes possible to further subdivide these groups on the basis of silicic acid column results or the difference in the fermentation of different substrates by different species within a group. The fermentation patterns varied from relatively simple patterns where acetic acid was the only end-product detected to more complex patterns where acetic, propionic, isobutyric, butyric, isovaleric and isocaproic were detected.

Lewis et al. (39) have also examined Clostridium species for volatile fatty acids. Comparison of their results with those previously discussed is difficult due to differences in growth media,

cultural techniques, operating conditions of the gas chromatograph and detector used. Moore et al. have carried out their study with a thermal conductivity detector which has been shown to be less sensitive than the flame ionization detector used by Lewis et al. However, no new or unidentified fatty acid was reported by Lewis et al. even though the fermentation patterns they obtained did not always resemble those obtained by Moore et al.

Henis et al. (22) demonstrated a variety of volatile fatty acids and neutral compounds in extracts of the growth medium of Escherichia coli, Aerobacter aerogenes, Pseudomonas aeruginosa and six species of Bacillus by employing both flame ionization and electron capture detectors. The peak area of each compound was measured and a letter assigned to each. The letters were then arranged in order corresponding to decreasing peak areas, thus establishing a signature for each bacterial strain. Differences in signatures allowed differentiation of genera, species and strains. O'Brien (52) carried out a similar study except that the bacterial signatures were derived by listing the peak areas according to increasing retention times. Peak areas cannot always be assumed to be an absolute measure of product concentration since area is dependent on a variety of factors including extraction efficiency, volatility and detector sensitivity. On the other hand, with O'Brien's technique different signatures would only be obtained if qualitative differences are observed.

The previous publications all describe methods of extracting metabolic products from liquid media using pure cultures. Cecchini

and O'Brien (12) described a procedure for detecting Escherichia coli in the presence of Aerobacter aerogenes by direct injection of culture supernatant on the GLC column. E. coli was shown to produce ethyl alcohol from lactose at 44°C whereas A. aerogenes did not. However, when cultured at 37°C with lactose or at 44°C with glucose, A. aerogenes will also produce ethanol.

Yoshioka et al. (76) have also analyzed bacterial volatile metabolites by direct analysis of the culture supernatants. They also differentiated between two closely related members of Enterobacteriaceae, namely Aerobacter aerogenes and Klebsiella pneumoniae. The former produced diacetyl from the medium used while the latter produced acetic acid.

O'Brien (52) has suggested another possible application of GLC end-product determination. When dealing with organisms that grow poorly in media used routinely, determination of fermentation of various sugars for purposes of identification often give negative results. If resting cells were suspended in buffered solutions containing various sugars and incubated for a certain length of time GLC analysis of the supernatant fluid could yield positive information. Control cells incubated in buffer alone would have to be used in order to eliminate the possibility that the end-products detected are not due to endogenous metabolism only.

Recently, Brooks and Moore (8) have developed GLC methods for the analysis of amines, long chain fatty acids and neutral products from culture supernatants of various species of Clostridia.

Cultures of 62 strains of Clostridia representing 13 species were divided into 7 groups on the basis of fatty acids and neutral products, into 10 groups on the basis of amine products, and into 12 groups on the basis of alcohol and neutral products. When considering all three types of end-products, they were divided into 13 groups. These results illustrate the fact that a single criterion cannot always be used for classification, but a group of criteria must be used.

The third GLC approach for the rapid characterization of microorganisms is that suggested by Reiner (57) as previously mentioned. Pyrolysis-gas-liquid chromatography (P-GLC) is a relatively simple technique in that the sample preparation consists of simply lyophilizing the bacteria and weighing the pellet before analysis. The dried bacteria are then pyrolyzed in an inert atmosphere yielding thermally degraded gaseous products which are then separated on an appropriate column and recorded as a series of peaks. The profile, or pyrogram as it is called, represents the unique chemical composition of the microorganism.

Reiner has published several applications of this technique for the differentiation of mycobacteria (58 and 60) and enteropathogenic bacteria (59). Cone and Lechowich (13) were able to differentiate between Clostridium botulinum types A, B and E by P-GLC. Not only did the pyrograms permit differentiation between types but also between spores and vegetative cells.

The disadvantages of this technique are the complexity of

the pyrograms and the lack of information as to the identity of the compounds separated.

Tentative identification of fatty acids or other components may be carried out by comparing the retention times of the unknown components with those of highly purified standards, and by calculation of carbon numbers and retention indices. However, misidentifications will occur due to failure of peak resolution. For positive identification, combined gas-chromatography-mass spectrometry has proven to be a very reliable technique. Oró et al. (54) have successfully characterized the aliphatic hydrocarbons and fatty acids of some marine and fresh-water microorganisms. Tornabene et al. (68) have identified the fatty acids and aliphatic hydrocarbons in Sarcina lutea by GLC and combined GLC-mass spectrometry.

II

CLASSIFICATION ON THE BASIS OF GAS-LIQUID CHROMATOGRAPHIC DETERMINATION OF CELLULAR FATTY ACIDS

METHODS AND MATERIALS

Twenty-six pure cultures of Gram-negative, non-spore-forming rods representing two families, three genera and eight species were chosen for this study (Tables II and III). They were obtained from six sources. Miss Y. Goodman (University of Alberta, Edmonton) supplied the Pasteurella multocida strains, and P. haemolytica. P. pseudotuberculosis was supplied by the Provincial Laboratory of Public Health, Edmonton, Alberta. The following strains were isolated from clinical materials at the University Hospital, Edmonton: Bacteroides melaninogenicus and the corroding Gram-negative rods tentatively designated Bacteroides corrodens: 4282 (Lane), 4053, S-209, 53P, 53W, 3936, and 4482. B. oralis was supplied by E. Bergen (University of Alberta, Edmonton). Dr. S. M. Finegold (Veterans Administration Hospital, Los Angeles, California) supplied the two strains of corroding bacilli B-912 and B-916. The corroding strain EDMH-1 was isolated from amniotic fluid at the Misericordia Hospital, Edmonton. B. corrodens 10596 (Henriksen's type strain 333/54-55), B. corrodens A40/68 and Haemophilus aphrophilus 5886 were obtained from the National Collection of Type Cultures, Colindale, London, England.

During the course of the study, the organisms were maintained on blood agar and modified Wahren and Holme medium. Transfers were made at weekly intervals, incubated anaerobically for 3 days and then stored at 4 °C. The strains were also lyophilized in serum for long-term storage.

TABLE II

BACTEROIDES CULTURES AND STRAINS OF CORRODING BACILLI OF
UNCERTAIN TAXONOMIC STATUS USED IN THIS STUDY^a

Genus	Species	Original source
<u>Bacteroides</u>	<u>fragilis</u>	NCTC 9343
	<u>oralis</u>	Bergen, E.
	<u>melaninogenicus</u>	UAH
	<u>corrodens</u>	NCTC 10596 (333/54-55)
	<u>corrodens</u>	NCTC A40/68
Unidentified corroding strains		B-916 (Finegold)
		53P (UAH-sputum)
		53W (variant of 53P)
		S-209 (UAH - sputum)
		3936 (UAH - sputum)
		4482 (UAH - neck lesion)
		EDMH-1 (Misericordia Hospital - amniotic fluid)
		B-912 (Finegold)
		4053 (UAH - heel lesion)
		4282 (Lane) (UAH - face lesion)

a Unidentified corroding strains were tentatively designated Bacteroides corrodens, following current usage.

TABLE III
BRUCELLACEAE CULTURES USED IN THIS STUDY FOR COMPARISON PURPOSES

Genus	Species	Original source
<u>Pasteurella</u>	<u>multocida</u> A ^a	10456-56 (UAH - human sputum)
	<u>multocida</u> A	5568-61 (UAH - human sputum)
	<u>multocida</u> D	675-59 (UAH - human sputum)
	<u>multocida</u> D	10955-55 (UAH - human CSF ^b)
	<u>multocida</u> NT	5228-58 (UAH - cat bite)
	<u>multocida</u> NT	MU3004-58 (UAH - human peritonitis)
	<u>multocida</u> NT	M191-60 (UAH - dog bite)
	<u>multocida</u> C	(dog)
	<u>haemolytica</u>	M1583-62 (goat)
	<u>pseudotuberculosis</u>	Provincial Laboratory of Public Health stock culture
<u>Haemophilus</u>	<u>aphrophilus</u>	NCTC 5886

^a Letter following species name refers to serotype; NT = non-typable.

^b Cerebrospinal fluid.

TABLE IV
BIOCHEMICAL CHARACTERISTICS OF THE STRAINS OF CORRODING
BACILLI AND OF THREE BACTEROIDES SPECIES

	catalase	urease	arginine decarboxylase	lysine decarboxylase	ornithine decarboxylase	aesculin hydrolysis	starch hydrolysis	nitrate reduction	oxidase (i) ^a	oxidase (ii) ^b
Group I										
<u>B. corrodens</u> NCTC 10596 (333/54-55)	-	-	+	+	+	-	-	+	+	+
<u>B. corrodens</u> NCTC A40/68	-	-	+	+	+	-	-	+	+	+
B-916	-	-	+	+	+	-	-	+	+	+
S-209	-	-	+	+	+	-	-	+	+	+
53P	-	-	+	+	+	-	-	+	+	+
53W	-	-	+	+	+	-	-	+	+	+
Group II										
B-912	-	+	-	-	-	-	-	+	+	+
EDMH-1	-	+	-	-	-	-	-	+	+	+
4053	-	+	-	-	-	-	-	+	+	+
4282 (Lane)	-	+	-	-	-	-	-	+	+	+
Group III										
3936	-	-	+	-	+	+	+	-	-	+
4482	-	-	+	-	v	+	+	-	-	+
<u>B. fragilis</u>	+	-	-	+	-	+	-	-	-	-
<u>B. oralis</u>	+	-	+	-	v	+	-	-	-	-
<u>B. melaninogenicus</u>	+	-	+	-	-	-	-	-	-	-

^a (i) dimethylparaphenylenediamine

v = variable

^b (ii) tetramethylparaphenylenediamine

Growth medium for fatty acid analysis

The culture medium used to grow the organisms for the isolation of fatty acids was prepared according to Wahren and Holme (72) with some modifications. L-cystine was reduced from 0.05% to 0.005% because growth of some of the corroding bacilli was inhibited by the higher concentration of this amino acid. Methylene blue was omitted because the facultative strains of corroding bacilli were inhibited by its presence. As the corroding bacilli grew very poorly in liquid media, 1.5% agar was added and the organisms were grown on solid medium in Petri dishes. Stirred fermenters used by Wahren and Holme are not available in this laboratory. Growth was stimulated by the addition of 1.012 M NaHCO_3 as described by Gibbons and MacDonald (19). It also served to buffer this same medium in the liquid form for end-product determinations. From trial experiments with liquid media, a slightly better growth was achieved when the medium was buffered.

Cultural procedures

Cultures maintained on Wahren and Holme modified medium were subcultured onto 50 to 100 plates of this medium. They were incubated anaerobically in Baird and Tatlock cold catalyst jars which were evacuated and filled with a mixture of 90% H_2 and 10% CO_2 and incubated at 37°C. The Bacteroides and corroding strains were incubated for 72 hours and the Pasteurella strains for 48 hours. At the end of the growth period, the cells were removed from the

plates by washing with 0.04M phosphate buffered saline (pH 7.2). The cell suspension was centrifuged at 8000 rpm for 5 minutes and washed twice with buffered saline. The final suspension was centrifuged at 12,000 rpm for 10 minutes, the supernatant removed and the packed cells stored at -20°C until extracted. Preliminary studies indicated the presence of trace amounts of fatty acids in 1 g of growth medium. This was not considered of sufficient magnitude to affect the results reported in this study since during the harvesting procedure, trace amounts only of agar may have been incorporated in the bacterial pellet. Additionally, of the seven small peaks present in this chromatogram, only two corresponded to fatty acids in the profiles of the organisms under study, hence the possible concentration of the components represented by these peaks is ruled out.

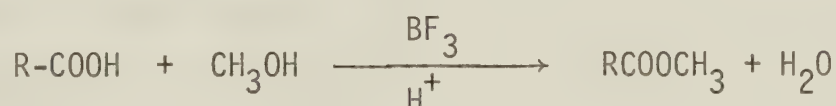
Principles underlying techniques used

Gas-liquid chromatography is another form of partition chromatography where the stationary phase is a liquid coated on a finely divided, inert, solid support packed into a column or, in the case of capillary columns, the liquid is coated on the walls of the column, and the mobile phase is a gas such as helium or nitrogen. This form of chromatography is applicable to the separation and measurement of compounds that are volatile or can be converted to volatile derivatives under the GLC conditions used.

The mixture to be chromatographed is flash-evaporated upon injection into a heated column; the volatilized components are

swept through the column by a constantly-flowing stream of gas. As the individual components emerge from the column at rates determined by their affinity for the stationary phase, they pass through a detection device and the data is recorded on a chart as a series of peaks. The area under each peak is proportional to the concentration of the particular component in the mixture. Tentative identification of components may be achieved by the use of standards and/or combination of various liquid phases such as lubricating greases, silicone oils or polyesters of high molecular weight alcohols and dibasic acids. Several types of detectors, as previously discussed, are used. The choice of detector and liquid phase varies with the mixtures being analyzed.

Application of this technique to fatty acid analysis involves the use of volatile derivatives of fatty acids such as methyl esters. In this study the fatty acids were first extracted from the bacterial cells by a process of simultaneous hydrolysis and saponification to produce water-soluble potassium salts of the fatty acids. The fatty acid salts were converted to free fatty acids upon acidification and these were subsequently recovered from the aqueous phase by extraction with n-hexane. The fatty acids were converted to methyl esters with boron trifluoride methanol ($\text{BF}_3\text{-CH}_3\text{OH}$) as follows



Combined gas chromatography-mass spectrometry makes use of the highly effective separation of the components of a mixture by GLC and the positive identification of these components by the mass spectrometer. Each component, as it emerges from the GLC column may be trapped or analyzed directly by the mass spectrometer. A beam of gaseous ions from the sample is produced which is driven down a tube by the application of an electrostatic field. The beam is diffracted and focused by a magnetic prism so that the ions are sorted out according to their mass-to-charge ratios. The output signals thus produced are measures of the relative abundance of each ionic species present.

Extraction of fatty acids and preparation of methyl esters

The extraction technique is essentially that described by Kaneda (31). Packed cells were thawed, and weighed amounts (0.5 to 1.5 g) were suspended in 25 ml of 1% methanolic KOH solution and refluxed at 68°C for 4 hours. The resulting suspension was centrifuged at 3000 rpm for 15 minutes at 4°C. The supernatant was extracted three times with 15 ml of n-hexane (Raylo Chemicals, Edmonton) - redistilled and chromatographed prior to use - to remove neutral and basic materials; the aqueous phase was acidified to pH 2.0 with concentrated HCl and extracted four times with 15 ml of n-hexane. The acidified extracts containing the fatty acids were pooled and evaporated to 0.5 ml by means of a rotary flash evaporator (Buchler Instruments). The fatty acids thus isolated were

methyated in the following manner: 1 ml of boron trifluoride in methanol (14% w/v, Applied Science Laboratories) was added to the solution of fatty acids and heated for 2 minutes in a boiling water bath, after which 4 ml of distilled water were added and the aqueous mixture extracted three times with 10 ml of n-hexane. The extracts were combined and evaporated to approximately 0.1 ml by means of a flash evaporator. The fatty acid methyl esters were stored in a teflon-capped glass vial at -20°C until chromatographed.

All glassware used was cleaned with dichromic acid and thoroughly rinsed with distilled water. Control runs were performed on all materials and reagents used to ensure that they did not contribute in any way to the peaks of the GLC profiles.

GLC analysis

The fatty acid methyl esters were analyzed by use of a Carlo Erba Fractovap Model GV dual column gas chromatograph equipped with a hydrogen-flame ionization detector. The output was recorded on a Honeywell strip chart recorder and integrated on an Infotronics CR 100 digital integrator.

Separation was effected on a 3m silicone rubber gum column [3% SE30 on Chromosorb G. AW-DMCS, 60/70 mesh (Chromatographic Specialties, Brockville, Ontario) packed in 6 mm outer diameter stainless steel tubing.]

Operating parameters of the instrument were: injection temperature, 260°C; detector temperature, 280°C; column temperature

linearly programmed from 150°C to 250°C at 3°C per minute; carrier gas, nitrogen at 50 ml per minute; hydrogen adjusted to give maximum sensitivity; air, 300 ml per minute; input and output attenuation, 10^2 and 16 respectively; chart speed, one half-inch per minute.

For routine analysis 3 to 4 μ l of the methyl esters were analyzed for 33 minutes after injection under the above stated conditions. This time interval was sufficient to detect straight chain saturated and unsaturated fatty acid methyl esters of 6 to 22 carbon atoms in length (Fig. 1). Fatty acid quantities were determined from integrator data and the percentage of each acid was calculated from the ratio of the quantity represented by this peak to the total amount of all the peaks.

In this study, only those acids which were present at levels greater than 1% are considered, even though numerous smaller peaks were noted in several profiles.

For purposes of illustration of the profiles in this dissertation 2 to 3 μ l of sample were analyzed as described above, except that a logarithmic scale was used rather than a linear scale in order to show all of the peaks entirely; the attenuation was reduced to 10^2 and 8 and the chart speed to one-quarter inch per minute.

For identification purposes, the samples were also analyzed on a 7.5 m diethylene glycol succinate column [6% DEGS on Chromosorb G, AW-DMCS, 60/70 mesh (Chromatographic Specialties, Brockville, Ontario) packed in 4.7 mm outer diameter copper tubing].

- | | |
|---------------------------------|--|
| 1. methyl caproate plus solvent | 8. methyl laurate |
| 2. methyl heptanoate | 9. methyl myristate |
| 3. methyl caprylate | 10. methyl palmitate |
| 4. methyl nonanoate | 11. methyl oleate and methyl linoleate |
| 5. methyl caprate | 12. methyl stearate |
| 6. methyl undecylenate | 13. methyl arachidate |
| 7. methyl undecanoate | 14. methyl behenate |

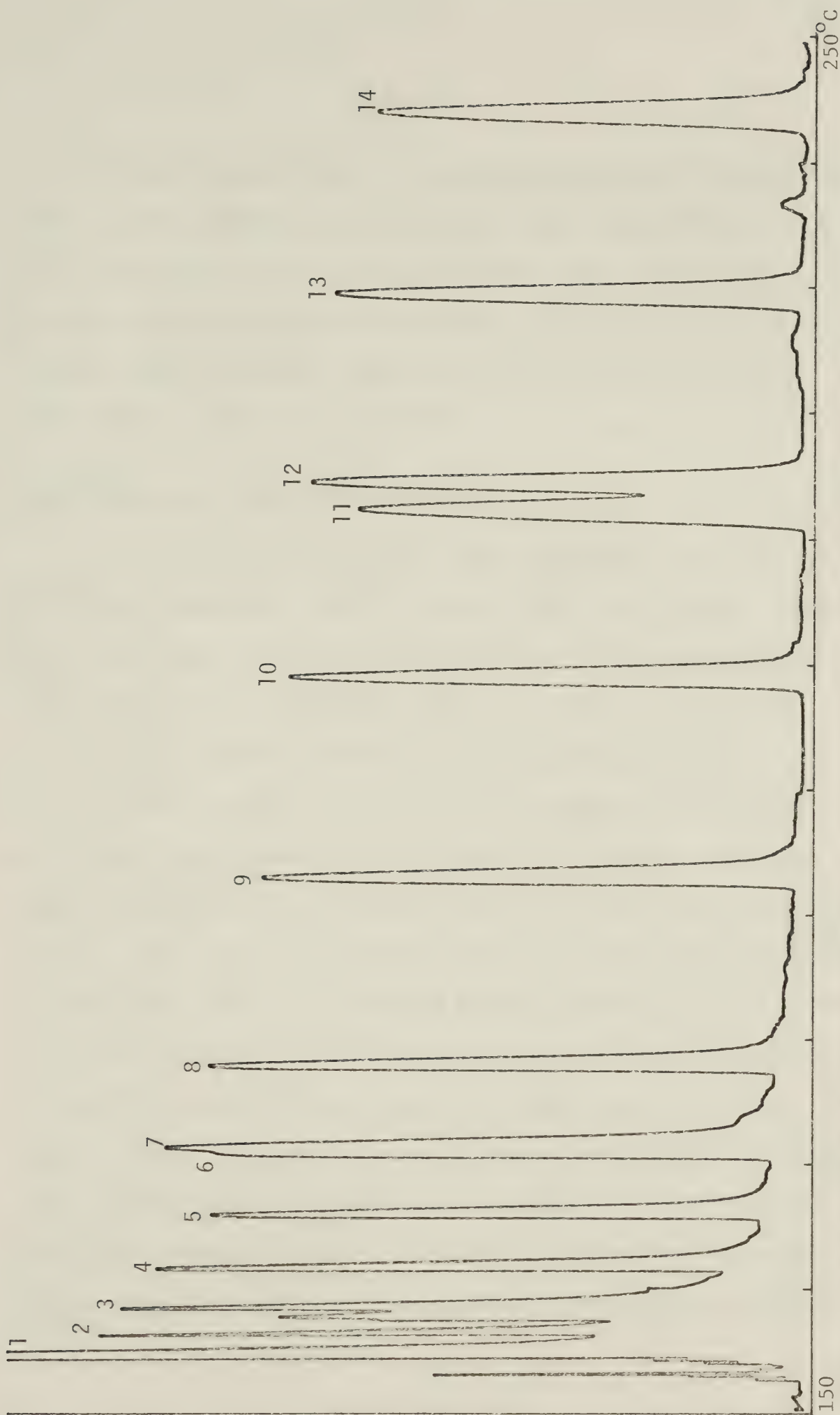


Fig. 1. GLC profile of fatty acid methyl ester standards. Numbers on peaks refer to identity of these standards.

The operating parameters were: injection and detector temperatures, 220°C; column temperature linearly programmed from 150°C to 200°C at 3°C per minute; carrier gas, nitrogen at 40 ml per minute; hydrogen adjusted to give maximum sensitivity; air, 300 ml per minute; input and output attenuation, 10^2 and 16 respectively; chart speed one half-inch per minute.

Identification of fatty acid methyl esters

The fatty acid methyl esters were tentatively identified by: (i) direct comparison of their retention times with standard fatty acid methyl esters (Applied Science Laboratories) chromatographed under identical GLC conditions. (Fig. 1 - note that $C_{11:0}$ and $C_{11:1}$ are not completely resolved from one another, and that $C_{18:1}$ and $C_{18:2}$ were eluted as one peak.); (ii) chromatography on both polar (DEGS) and nonpolar (SE-30) columns to identify unsaturated compounds by the shift in their position relative to saturated compounds. (On a non-polar stationary phase, an unsaturated component will be eluted before its saturated homolog whereas on a polar phase it will be retarded.); (iii) chromatography of bromo derivatives (on SE-30) to confirm identification of unsaturated fatty acids. (When the total fatty acid mixture is treated with Br_2 in cold ethyl ether (27) and then chromatographed, peaks due to unsaturated compounds will disappear from their original positions and new peaks corresponding to brominated fatty acids will be observed at much higher retention temperatures).

Positive identification of some of the major fatty acids was accomplished by combined gas chromatography-mass spectrometry. (Mass spectral scans were done by the Chemistry Department of the University of Alberta.) It was not possible to analyze the fragmentation patterns of all components because they were either present in amounts too small to be properly identified or gave complex mass spectral data for which no characteristic fragmentation patterns were available from the literature consulted (14). On the other hand, it was considered beyond the scope of the objectives of this study to identify completely all components present in the various fatty acid methyl ester profiles.

Those components for which identification was not possible will be referred to by retention values, or more specifically by 'carbon number' or 'equivalent chain length' (ECL) (21). This system of describing retention values relates the retentions of unknown fatty acids to those of normal saturated fatty acids where each acid is assigned a carbon number or ECL equal to its number of carbon atoms: for example, palmitic acid, $C_{15}H_{31}COOH$, has an ECL of 16.00. The ECL of unknown fatty acids is obtained by interpolation of the logarithms of the net isothermal retention volumes of the unknown and of the reference fatty acid methyl esters. An application of ECL or 'carbon number' to programmed temperature gas chromatography (PTGC) which involves a linear interpolation of retention temperatures is described by the authors:

$$C_X = C_A + \left(\frac{TRX - TRA}{TRB - TRA} \right) (C_B - C_A)$$

where C is ECL or "carbon number", X is the unknown. A and B are normal saturated fatty acids and T_R is retention temperature.

This method of using PTGC has the advantage of being able to produce relatively sharp symmetrical peaks along the entire length of the profile which is not always possible in isothermal chromatography where peaks at the end of the profile are often broadened considerably, so that they appear as flat peaks.

The information derived by ECL is essentially identical with that obtained by relative retention values where the retention volume or retention time of an unknown component is compared with that of a known compound. ECL may have some structural significance since the reference compounds are of the same chemical nature as the unknown compounds. For example, the ECL of linolenic acid $C_{17}H_{29}COOH$, as its methyl ester on Apiezon L is 17.55 (21): its normal saturated homolog, stearic acid has been assigned an ECL equal to its number of carbon atoms (18.00).

All ECL determinations were carried out under the GLC conditions previously described for obtaining quantitative data on the fatty acid methyl esters in each sample.

In the following discussion of the results obtained in this study the fatty acid methyl ester peaks will be referred to first by the peak letter as it appears on the chromatograms (Figs.2 to 19), followed by the identity of the peak where positive identification was possible, or, by ECL where identification was not possible. For example: peak D will be referred to as: D (lauric) acid;

similarly peak T as T (ECL = 16.83) acid.

On the chromatograms, the peaks labelled with upper case letters represent fatty acids present as 1% or more of the total fatty acids; peaks labelled with lower case letters represent less than 1% of the total fatty acids.

RESULTS

Reproducibility of results

Because of possible variation between replications of each culture, duplicate determinations were made. Two replicates from each bacterial strain were carried through the entire growth, harvest, extraction and methylation procedures individually and chromatographed separately. Results of duplicate experiments with several strains are shown in Table V. In general, identical chromatograms were obtained from both replicates. The range for an individual peak for a particular organism varied from 0 to 4% in 96% of the cases. It varied from 4 to 6% in 2% of the cases and from 6 to 8% in 2% of the cases. The greatest single variation was 13.6% which occurred in the peak labelled K on the chromatogram of B. fragilis (Fig. 9). In Tables V to XIII, the percentage shown for each fatty acid methyl ester is the arithmetic mean from duplicate cultures.

Grouping of strains

Visual comparisons of gas chromatographic methyl ester profiles of fatty acids extracted from the organisms under study permitted rapid separation into four groups. The strains were grouped without difficulty by observing the presence or absence of, and relative sizes of major peaks. Each group had certain major distinguishing fatty acid methyl ester peaks.

TABLE V
COMPARISON OF DUPLICATE ANALYSES OF THE PRINCIPAL FATTY
ACIDS OF SOME OF THE STRAINS UNDER STUDY

Peak ^a	Fatty acid ^b	Percentage of fatty acids					
		NCTC 10596		B-916		53P	
		I	II	I	II	I	II
D	12:0	4.4	4.3	5.7	6.3	5.3	4.7
G	(13.42)	tr ^c	tr	tr	tr	tr	tr
H	(13.71)	tr	tr	tr	tr	tr	tr
I	14:0	4.6	3.8	3.7	2.8	1.6	1.4
K	(14.72)	tr	tr	tr	tr	tr	1.0
L	15:0	tr	tr	tr	tr	tr	tr
P	16:1	18.4	17.2	18.0	18.1	22.7	20.4
Q	16:0	37.0	34.6	39.9	32.5	28.5	26.3
T	(16.83)	tr	tr	tr	tr	tr	tr
U	17:0	tr	tr	tr	tr	tr	tr
X	18:1	32.5	36.6	30.4	38.1	39.4	42.7
Y	18:0	2.5	2.4	1.3	1.2	1.6	1.8
B	(19.24)	tr	tr	tr	tr	tr	tr
C	(19.85)	tr	tr	tr	tr	tr	tr

^a Peak letter as indicated on chromatograms

^b Number to left of colon refers to number of carbon atoms; number to right refers to number of double bonds; numbers in brackets refer to ECL of unidentified fatty acids.

^c tr = less than 1%

Group I contained large percentages of both Q (palmitic) and X (octadecenoic) acids, with a small percentage of P (palmitoleic) acid, and relatively small amounts of D (lauric), I (myristic), and Y (stearic) acids. The most obvious distinguishing feature of this group compared with other groups was that Q (palmitic) and X (octadecenoic) acids were present in almost equal concentrations and that P (palmitoleic) acid was present in significant proportions, whereas it was present in only trace amounts in Groups II and III although it was a major peak in Group IV.

Group II was distinguished from other groups by the large percentage of X (octadecenoic) acid and smaller amounts of Q (palmitic), D (lauric), I (myristic) and Y (stearic) acids. It differed from Group I in that it had only one major peak and in this respect it resembled Group III.

Characteristic of Group III was its large percentage of K (ECL = 14.72) acid which appeared to be unique to this group. Other fatty acids such as E (ECL = 12.60), Q (palmitic), V (ECL = 17.48) and Y (stearic) acids were present in small amounts.

Group IV was characterized by large percentages of P (palmitoleic) and Q (palmitic) acids and a smaller percentage of I (myristic) acid. It resembled Group I in that it had two major fatty acid peaks but it differed from Group I in that its major fatty acid peaks were P (palmitoleic) and Q (palmitic) acids, whereas they were Q (palmitic) and X (octadecenoic) acids in Group I.

Quantitative comparisons of the percentages of the principal fatty acids from each strain supported the grouping made by visual observations.

The two type cultures of Bacteroides corrodens, NCTC 10596 (333/54-55) and NCTC A40/68, were placed in Group I, along with the facultatively anaerobic strains of corroding bacilli tentatively designated B. corrodens; B-916, 53P, 53W, and S-209. These strains had similar fatty acid methyl ester profiles which permitted them to be exclusively and homogeneously grouped. Some of the profiles are represented by Figs. 2 to 5. None of the six strains were of doubtful status in regard to grouping since each contained a high percentage of both Q (palmitic) and X (octadecenoic) acids. Differences in the relative proportions of these fatty acids among the strains may be noted. An indication of the range of differences between these two acids is represented in Table VI. Strains B. corrodens NCTC 10596 (333/54-55), NCTC A40/68, B-916, and S-209 contained almost equal percentages of Q (palmitic) and X (octadecenoic) acids, Q (palmitic) acid being present in a slightly higher concentration than X (octadecenoic) acid. Strains 53P and 53W differ from the other strains in Group I in that X (octadecenoic) acid was present in much greater concentration than Q (palmitic) acid; 41.4% and 46.4% respectively for X (octadecenoic) acid and 27.4% and 28.1% respectively for Q (palmitic) acid. However, there was no doubt that these two strains belonged to this group, since Group I was the only group in which Q (palmitic) and X (octadecenoic) acids were both

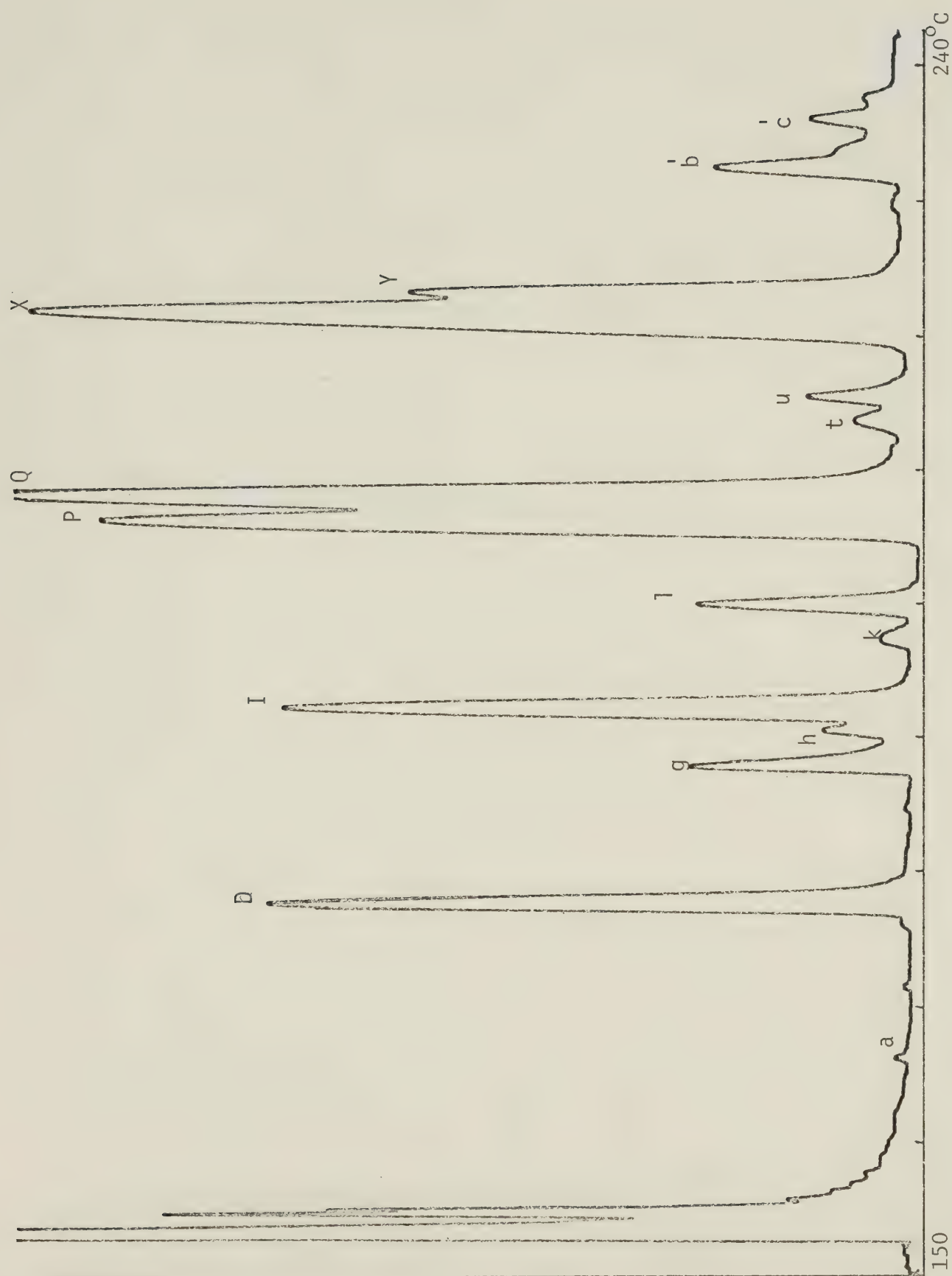


Fig. 2. GLC profile of methyl esters of fatty acids from *Bacteroides corrodens* NCTC 10596 (333/54-55). Letters on peaks refer to identity of acids as shown in Table VI; lower case letters represent trace amounts.

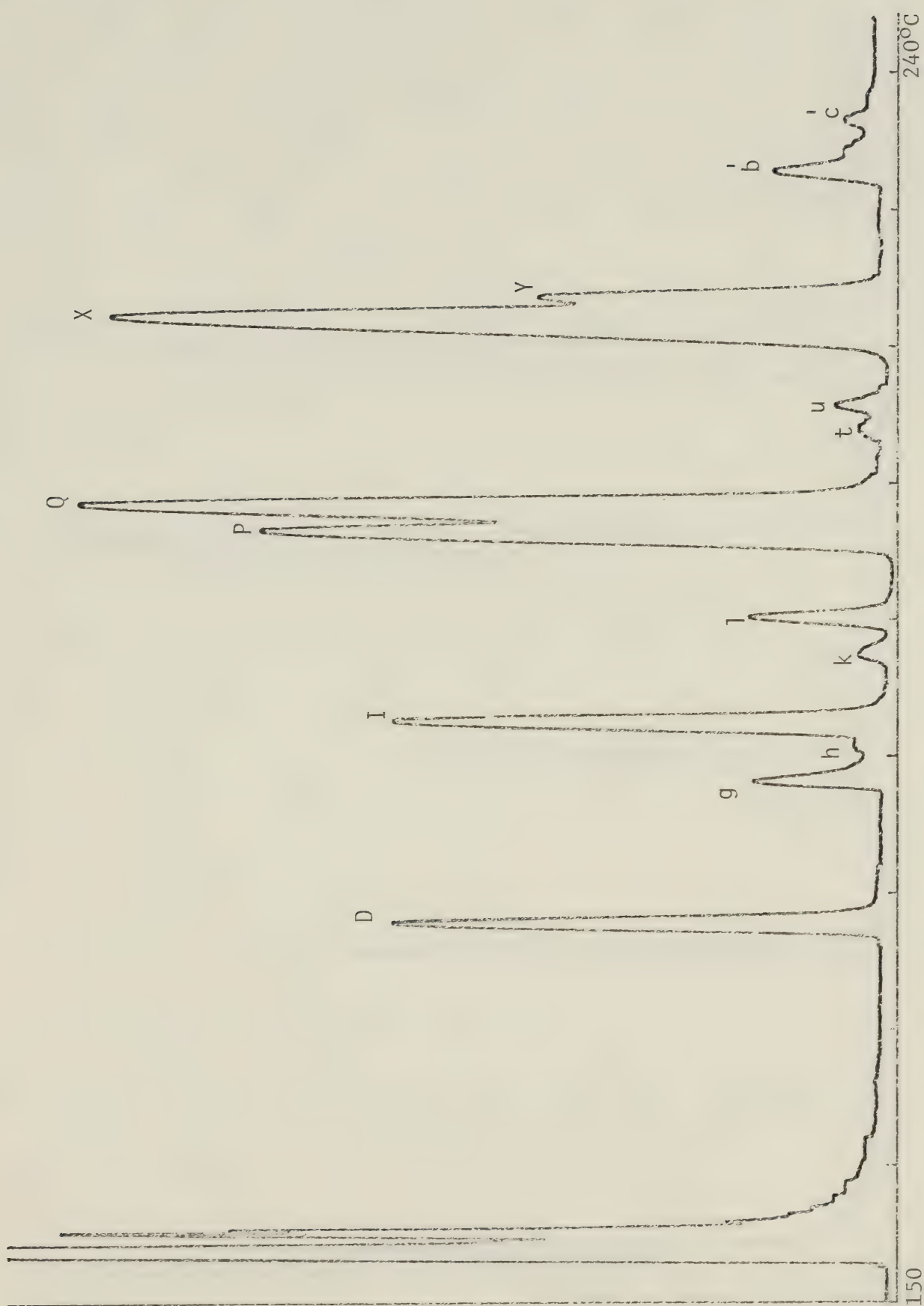


Fig. 3. GLC profile of methyl esters of fatty acids from *Bacteroides corrodens* NCTC A40/68. Letters on peaks refer to identity of acids as shown in Table VI; lower case letters represent trace amounts.

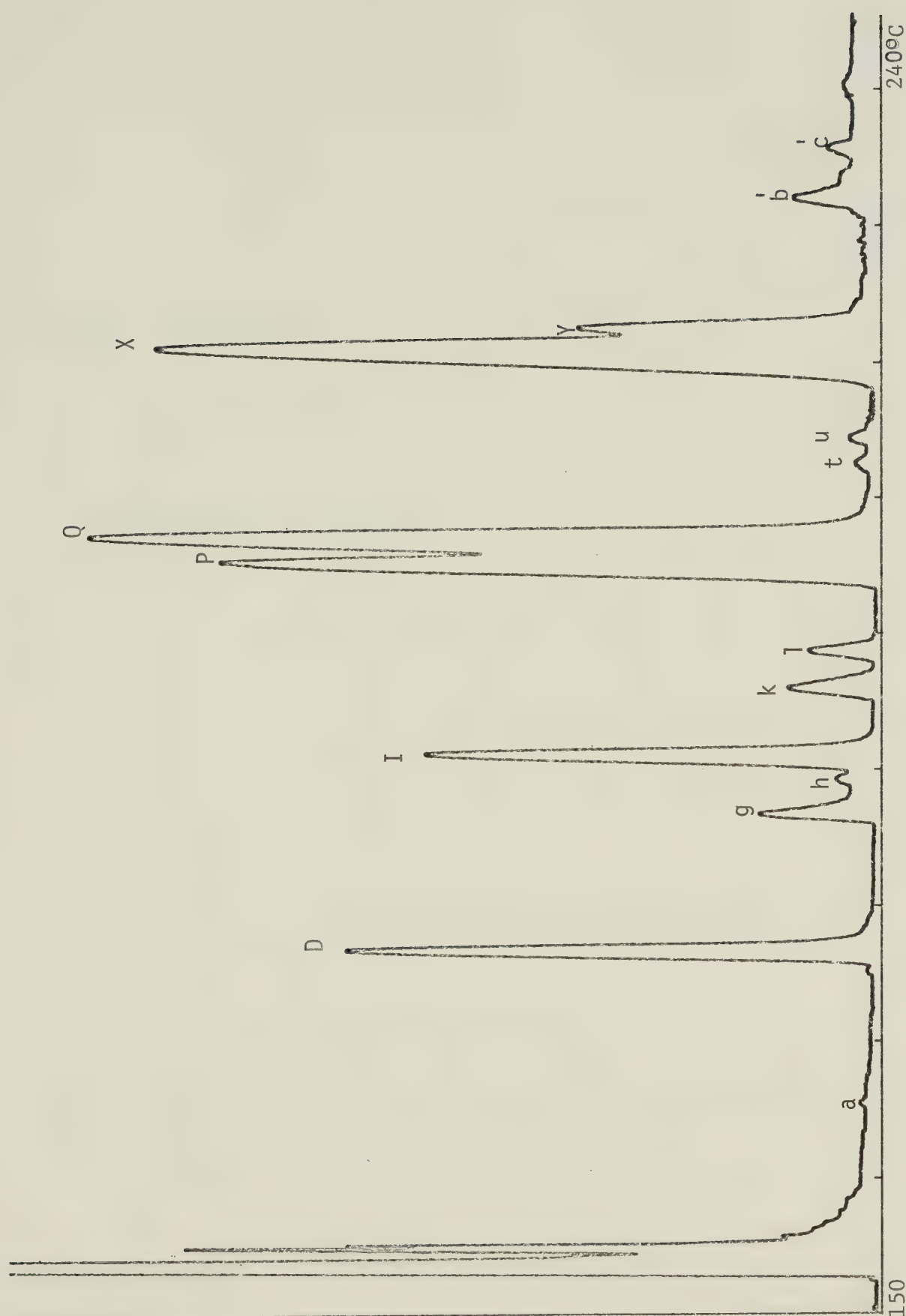


Fig. 4. GLC profile of methyl esters of fatty acids from corroding strain B-916. Letters on peaks refer to identity of acids as shown in Table VI; lower case letters represent trace amounts.

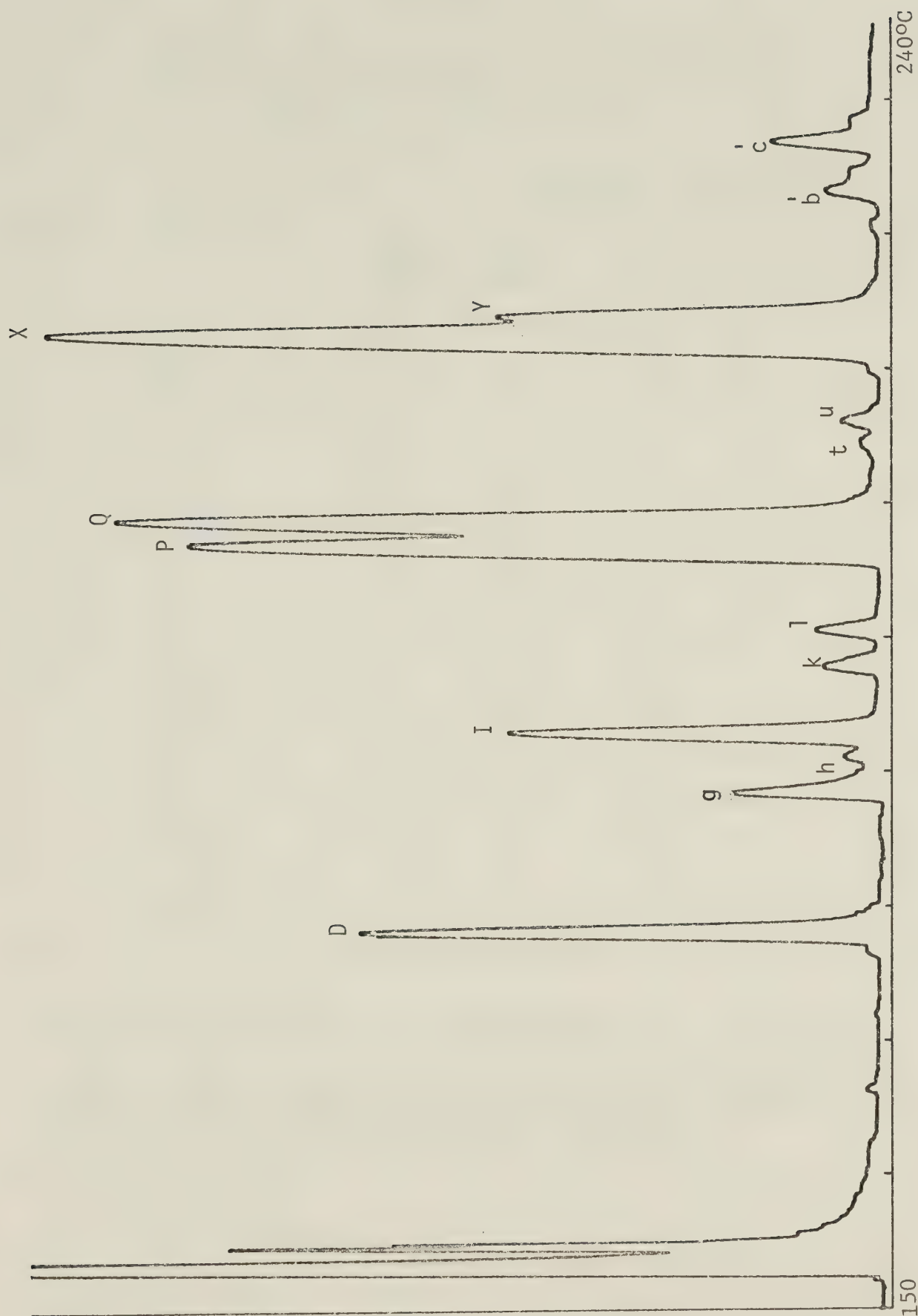


Fig. 5. GLC profile of methyl esters of fatty acids from corroding strain 53P. Letters on peaks refer to identity of acids as shown in Table VI; lower case letters represent trace amounts.

TABLE VI
COMPARISON OF THE FATTY ACID COMPOSITION OF GROUP I
STRAINS OF CORRODING BACILLI

Peak ^a	Fatty acid ^b	Percentage of fatty acids					
		NCTC 10596	NCTC A40/68	53P	53W	S209	B-916
A	-	-	-	tr ^c	-	-	-
D	12:0	4.4	3.8	5.0	3.8	4.4	6.0
G	(13.42)	tr	tr	tr	tr	tr	tr
H	(13.71)	tr	tr	tr	tr	tr	tr
I	14:0	4.2	3.8	1.5	1.4	1.4	3.2
K	(14.72)	tr	tr	tr	tr	tr	tr
L	15:0	tr	tr	tr	tr	tr	tr
P	16:1	17.8	14.3	21.6	18.0	21.2	18.1
Q	16:0	35.8	38.8	27.4	28.1	35.4	36.2
T	(16.83)	tr	tr	tr	tr	tr	tr
U	(17.02)	tr	tr	tr	tr	tr	tr
X	18:1	34.6	36.7	41.4	46.4	35.2	34.2
Y	18:0	2.4	1.4	1.7	1.8	1.7	1.2
B	(19.24)	tr	tr	tr	tr	tr	tr
C	(19.85)	tr	tr	tr	tr	tr	tr

^a Peak letter as indicated on chromatograms.

^b Number to left of the colon refers to the number of carbon atoms; number to right refers to number of double bonds; numbers in brackets refer to ECL of unidentified fatty acids.

^c tr = less than 1%

major components of the fatty acid spectrum. Strain 53W is a non-pitting variant of 53P. The next most abundant fatty acid of this group of organisms was P (palmitoleic) acid, which was present in concentrations varying from 14.3% to 21.6%. This fatty acid was detected in trace amounts only in the other strains of corroding bacilli. Other fatty acids present in low concentrations were D (lauric), I (myristic) and Y (stearic) acids plus several others in trace amounts only.

Cultures comprising Group II are the four anaerobic strains of corroding bacilli also tentatively designated B. *corrodens*, B-912, EDMH-1, 4053 and 4282 (Lane). These strains were easily grouped together on the basis of the large proportion of X (octadecenoic) acid (Figs.6 to 8). This fatty acid represented 70.4% to 74.9% of the total fatty acids (Table VII). The next most abundant fatty acid was Q (palmitic) acid which varied from 8.3% to 11.4%. The following fatty acids were generally of low and variable concentration: D (lauric), I (myristic) and Y (stearic) acids.

Group III contains three recognized species of the genus Bacteroides, namely B. *fragilis*, B. *oralis*, and B. *melaninogenicus* (Figs.9 to 11) and the two anaerobic strains of corroding bacilli 3936 (Fig. 12) and 4482. The greater concentration of K (ECL = 14.72) in these two strains of corroding bacilli did not prohibit the grouping of these organisms with recognized species of the genus Bacteroides since this fatty acid was unique to this group (Table VIII). The organisms included in this group are not as homogeneous

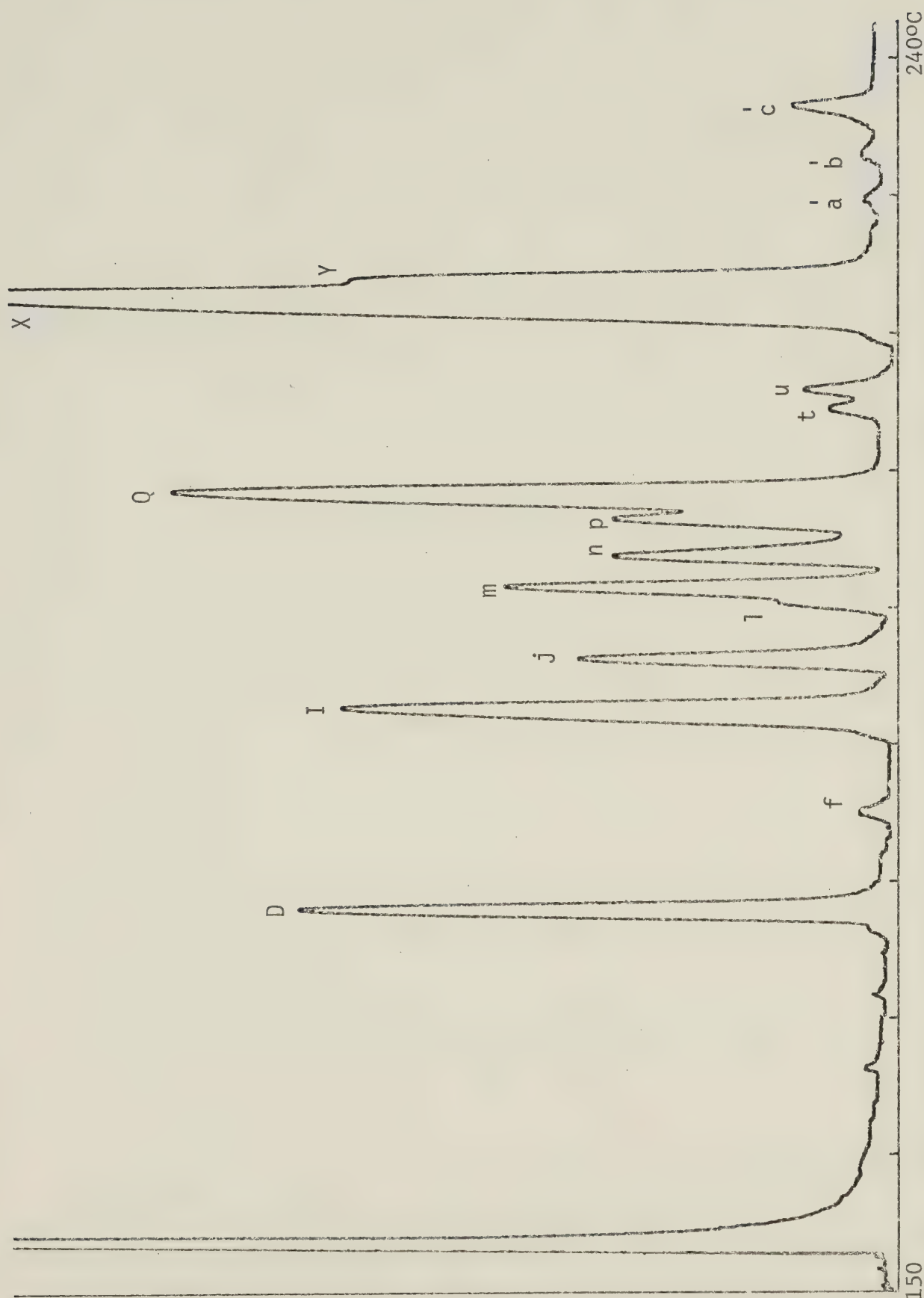


Fig. 6. GLC profile of methyl esters of fatty acids from corroding strain B-912. Letters on peaks refer to identity of acids as shown in Table VII; lower case letters represent trace amounts.

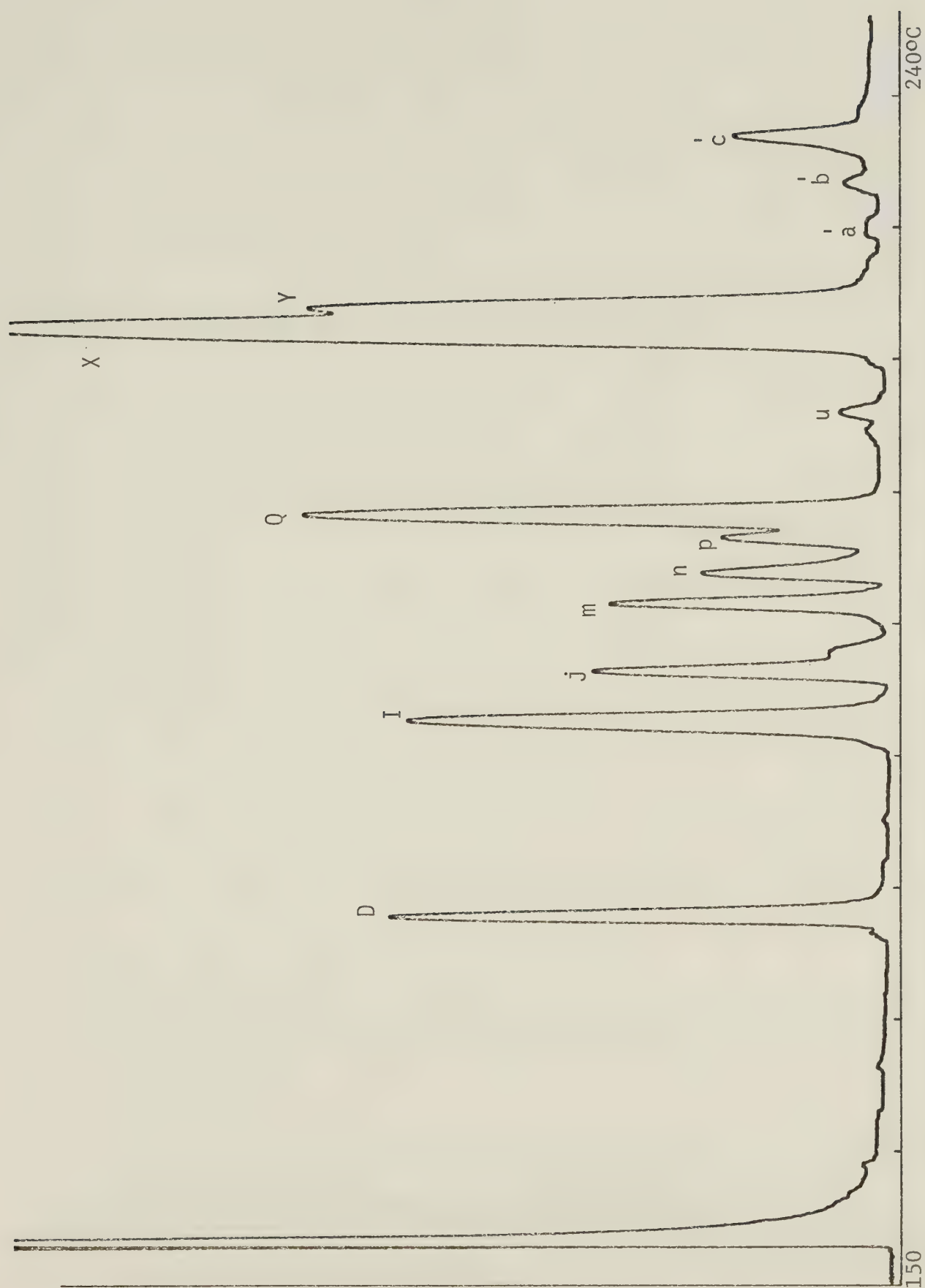


Fig. 7. GLC profile of methyl esters of fatty acids from corroding strain EDMH-1. Letters on peaks refer to identity of acids as shown in Table VII; lower case letters represent trace amounts.

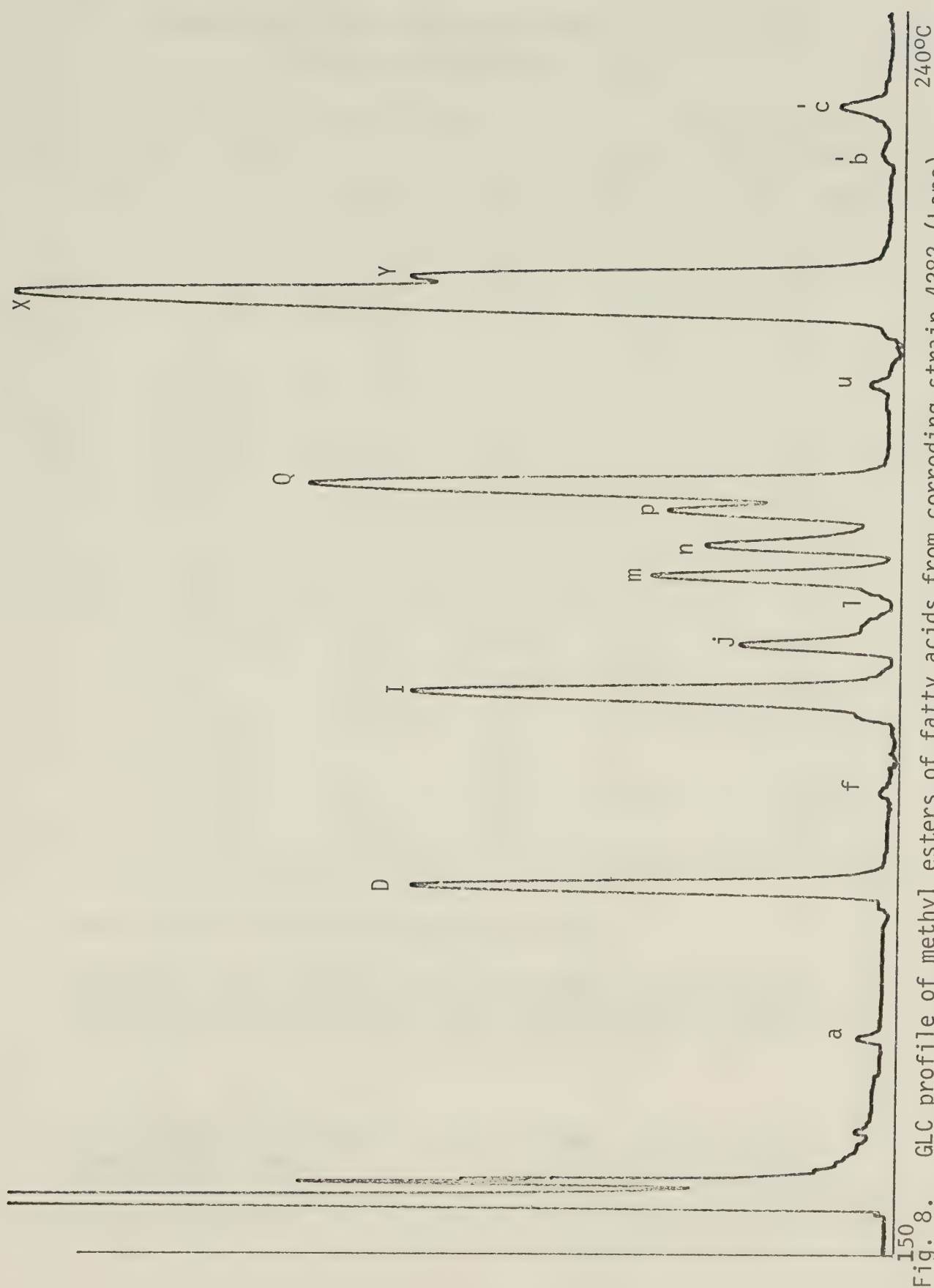


Fig. 8. GLC profile of methyl esters of fatty acids from corroding strain 4282 (Lane). Letters on peaks refer to identity of acids as shown in Table VII; lower case letters represent trace amounts.

TABLE VII
COMPARISON OF THE FATTY ACID COMPOSITION OF GROUP II
STRAINS OF CORRODING BACILLI

Peak ^a	Fatty acid ^b	Percentage of fatty acids			
		B-912	4053	EDMH-1	4282 (Lane)
A	-	tr ^c	tr	tr	tr
D	12:0	4.1	3.3	3.8	3.7
F		tr	tr	tr	tr
I	14:0	3.6	4.4	4.3	7.0
J	(14.50)	tr	tr	tr	tr
LM ^d	(15.14)	1.1	tr	tr	tr
N	(15.43)	tr	tr	tr	tr
P	16:1	tr	tr	tr	1.5
Q	16:0	11.4	9.1	8.3	10.8
U	17:0	tr	tr	tr	tr
X	18:1	74.9	72.2	72.8	70.4
Y	18:0	3.2	8.2	7.2	4.5
A	(18.84)	tr	tr	tr	-
B	(19.24)	tr	tr	tr	tr
C	(19.85)	tr	tr	tr	tr

^a Peak letter as indicated on chromatograms.

^b Number to left of colon refers to number of carbon atoms; number to right refers to number of double bonds; numbers in brackets refer to ECL of unidentified fatty acids.

^c tr = less than 1%

^d Not completely resolved from one another; M is the major contributing peak.

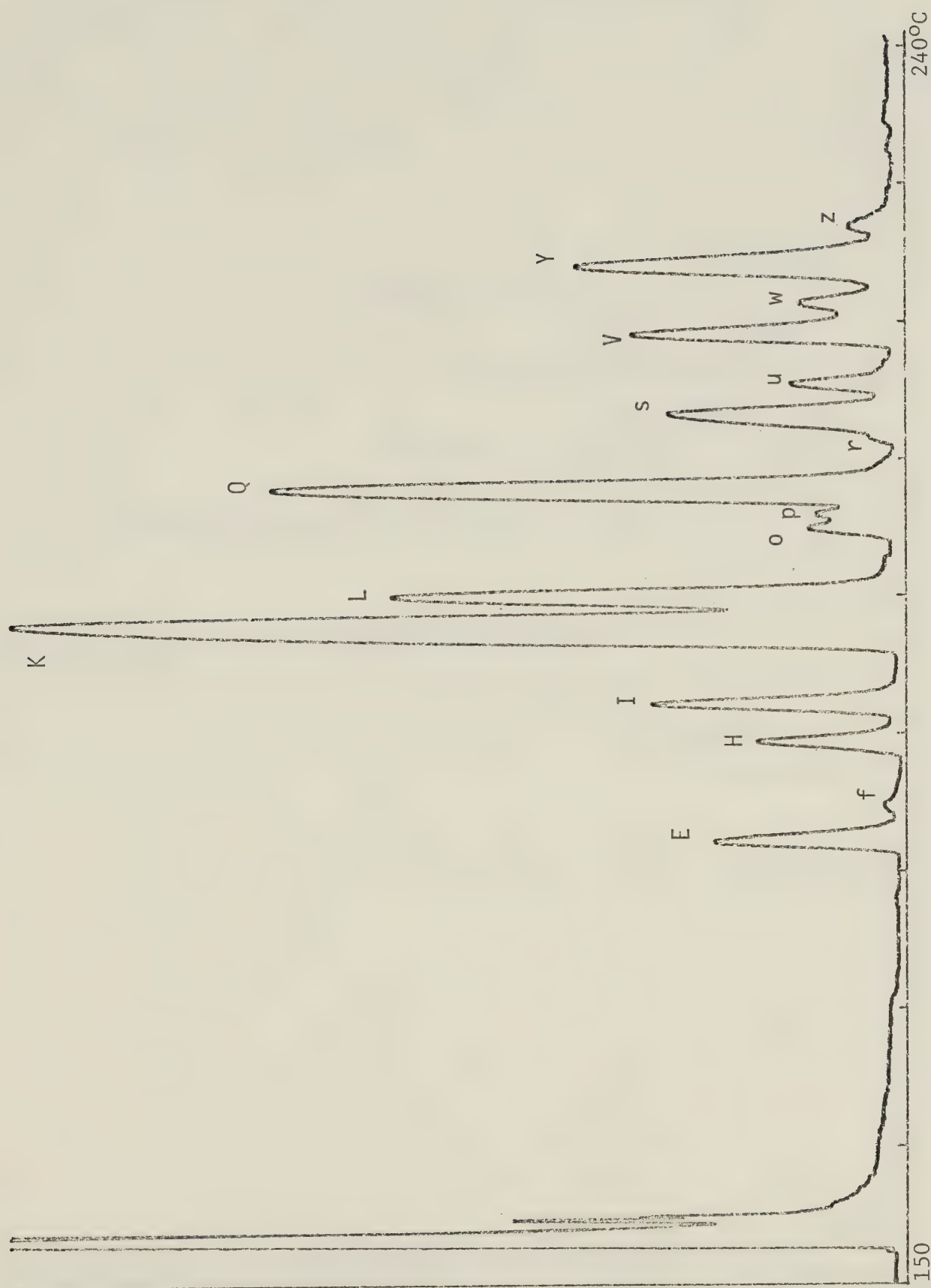


Fig. 9. GLC profile of methyl esters of fatty acids from *Bacteroides fragilis* NCTC 9343. Letters on peaks refer to identity of acids as shown in Table VIII; lower case letters represent trace amounts.

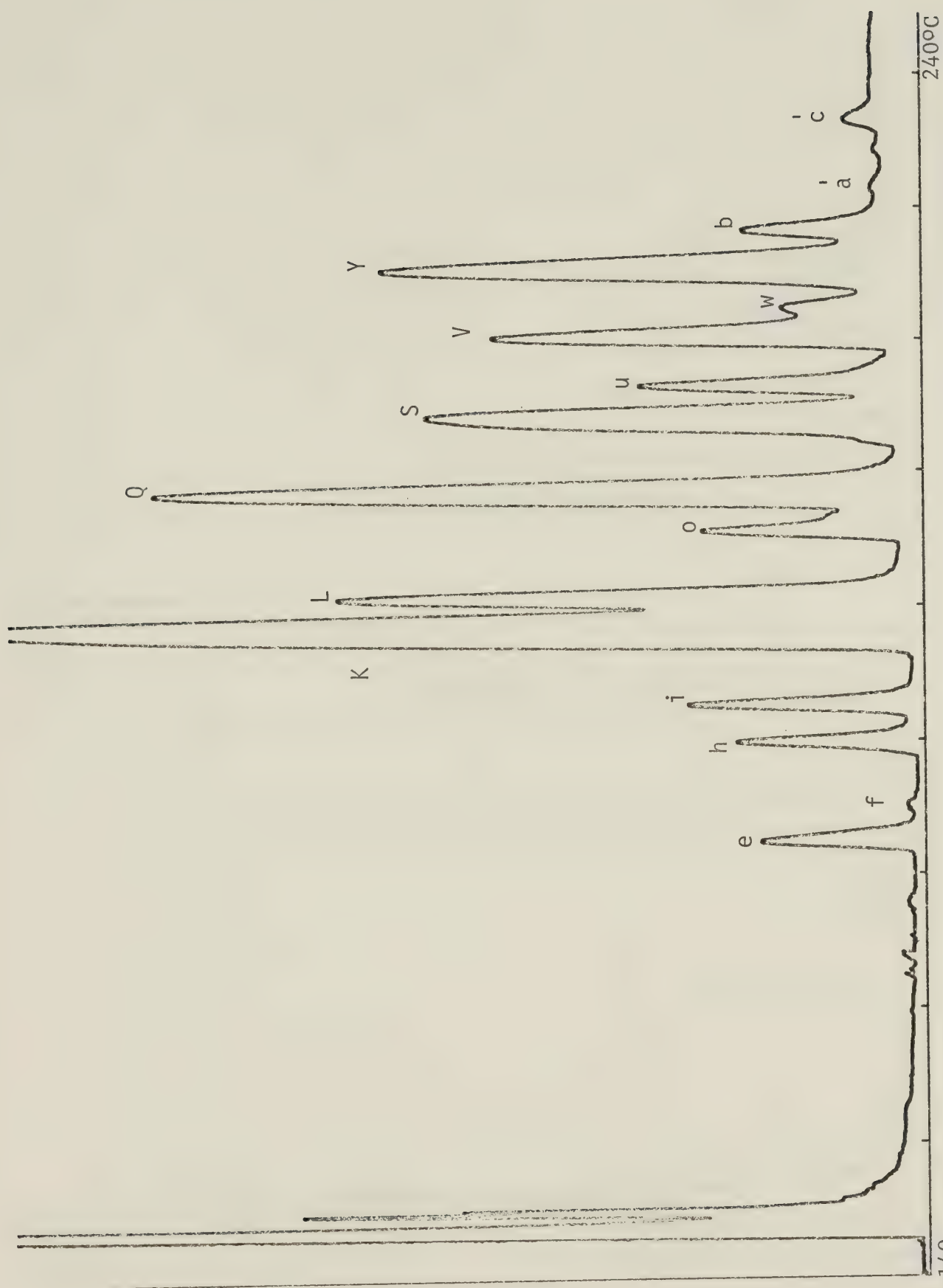


Fig. 10. GLC profile of methyl esters of fatty acids from *Bacteroides oralis*. Letters on peaks refer to identity of acids as shown in Table VIII; lower case letters represent trace amounts.

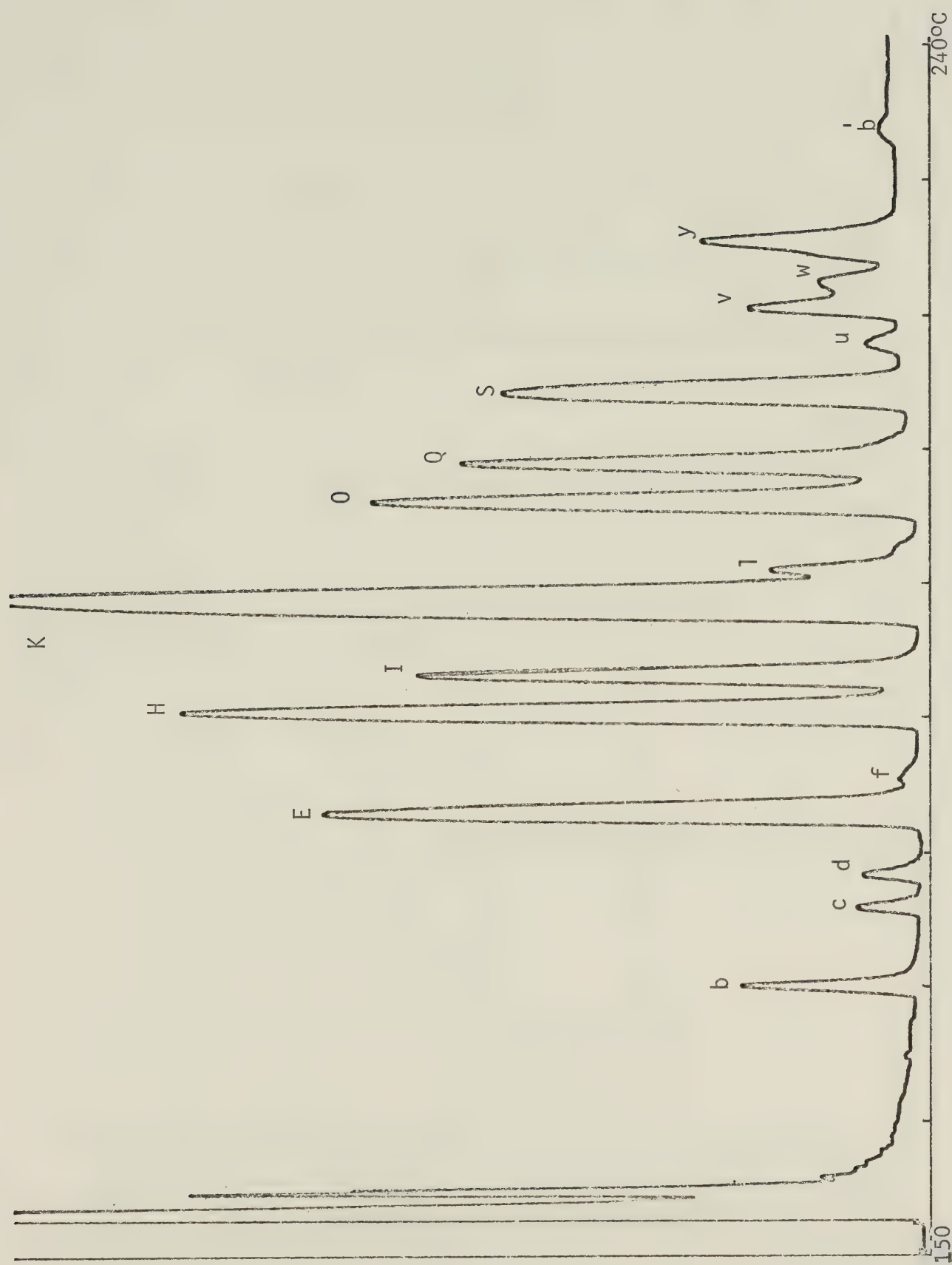


Fig. 11. GLC profile of methyl esters of fatty acids from *Bacteroides melaninogenicus*. Letters on peaks refer to identity of acids as shown in Table VIII; lower case letters represent trace amounts.

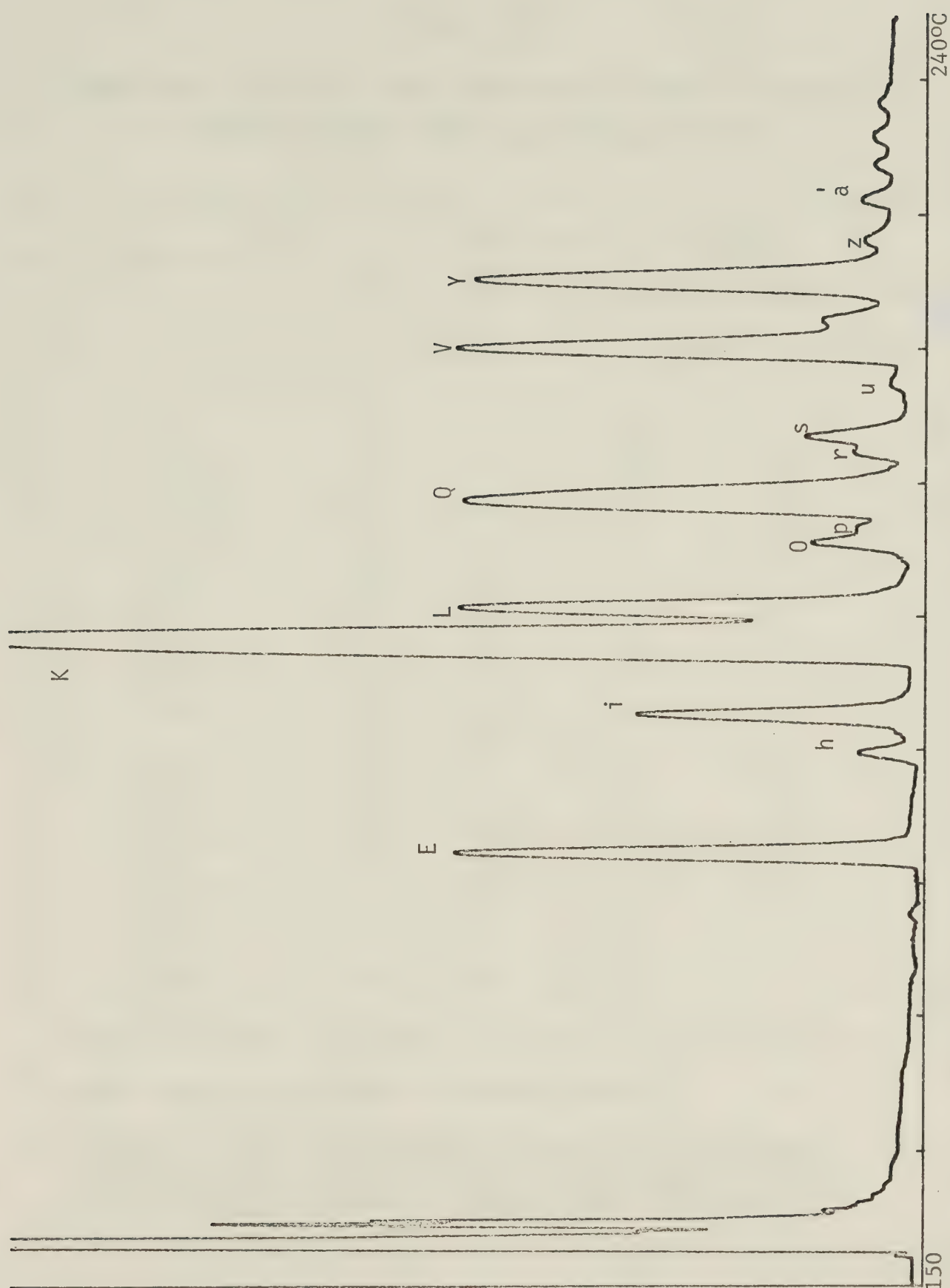


Fig. 12. GLC profile of methyl esters of fatty acids from corroding strain 3936. Letters on peaks refer to identity of acids as shown in Table VIII; lower case letters represent trace amounts.

TABLE VIII

COMPARISON OF THE FATTY ACID COMPOSITION OF GROUP III STRAINS
OF CORRODING BACILLI AND OF BACTEROIDES SPECIES

Peak ^a	Fatty acid ^b	Percentage of fatty acids				
		3936	4482	<u>B.fragilis</u>	<u>B.oralis</u>	<u>B.melanimo- genicus</u>
A	-	-	-	-	-	tr
B	-	-	-	-	-	tr
C	-	-	-	tr ^c	-	tr
D	12:0	-	-	tr	tr	tr
E	(12.60)	1.6	1.8	1.3	tr	7.6
F	-	-	-	tr	tr	tr
H	(13.71)	tr	tr	1.0	tr	15.9
I	14:0	tr	tr	2.1	tr	2.9
K	(14.72)	93.8	90.2	70.0	65.6	62.9
L	15:0	tr	1.0	9.8	5.2	tr
O	(15.64)	tr	tr	tr	tr	4.2
P	16:1	tr	tr	tr	tr	-
Q	16:0	1.4	2.6	10.8	16.0	2.9
R	-	tr	tr	-	-	-
S	(16.71)	tr	tr	tr	4.1	2.0
U	17:0	tr	-	tr	tr	tr
V	(17.48)	1.3	2.2	1.5	2.4	tr
W	(17.74)	tr	tr	tr	tr	tr
Y	18:0	tr	1.0	2.1	4.3	tr
Z	(18.52)	tr	tr	tr	tr	-
A	(18.84)	tr	tr	-	-	-
B	(19.24)	tr	tr	tr	-	tr
C	(19.85)	-	-	-	-	tr

^a Peak letter as indicated on chromatograms

^b Number to left of colon refers to number of carbon atoms; number to right refers to number of double bonds; numbers in brackets refer to ECL of unidentified fatty acids.

^c tr = less than 1%.

in their fatty acid compositions as those in Groups I and II. Greater variation in percentage composition of several fatty acids was encountered. For example, Q (palmitic) acid constitutes 10.8% and 16.0% respectively of the total fatty acids of B. fragilis and B. oralis, whereas it represented 2% or less of the total fatty acids of B. melaninogenicus, 3936 and 4482. On the other hand, B. melaninogenicus contained 15.9% of H (ECL = 13.71) acid, whereas it represented 1% or less of the total fatty acids of the other strains in this group. Moreover, B. melaninogenicus contained relatively important concentrations of E (ECL = 12.60) and O (ECL = 15.64) which were present in much lower concentrations in the other strains.

All the Pasteurella strains included in this study for purposes of comparison are included in Group IV. Representative profiles from each serotype of P. multocida are illustrated in Figs. 13 to 16. P. haemolytica and P. pseudotuberculosis fatty acid spectra are shown in Figs. 17 and 18 respectively. This group was characterized by large and almost equal percentages of P (palmitoleic) and Q (palmitic) acids. Generally the next most abundant fatty acid was I (myristic) acid and this was followed by varying amounts of X (octadecenoic) and Y (stearic) acids (Table IX). No qualitative differences in the fatty acid composition among the different serotypes of P. multocida strains were evident. However, some minor quantitative differences were seen. For example, the two major fatty acids of the serotypes A and D were Q (palmitic) and P (palmitoleic)

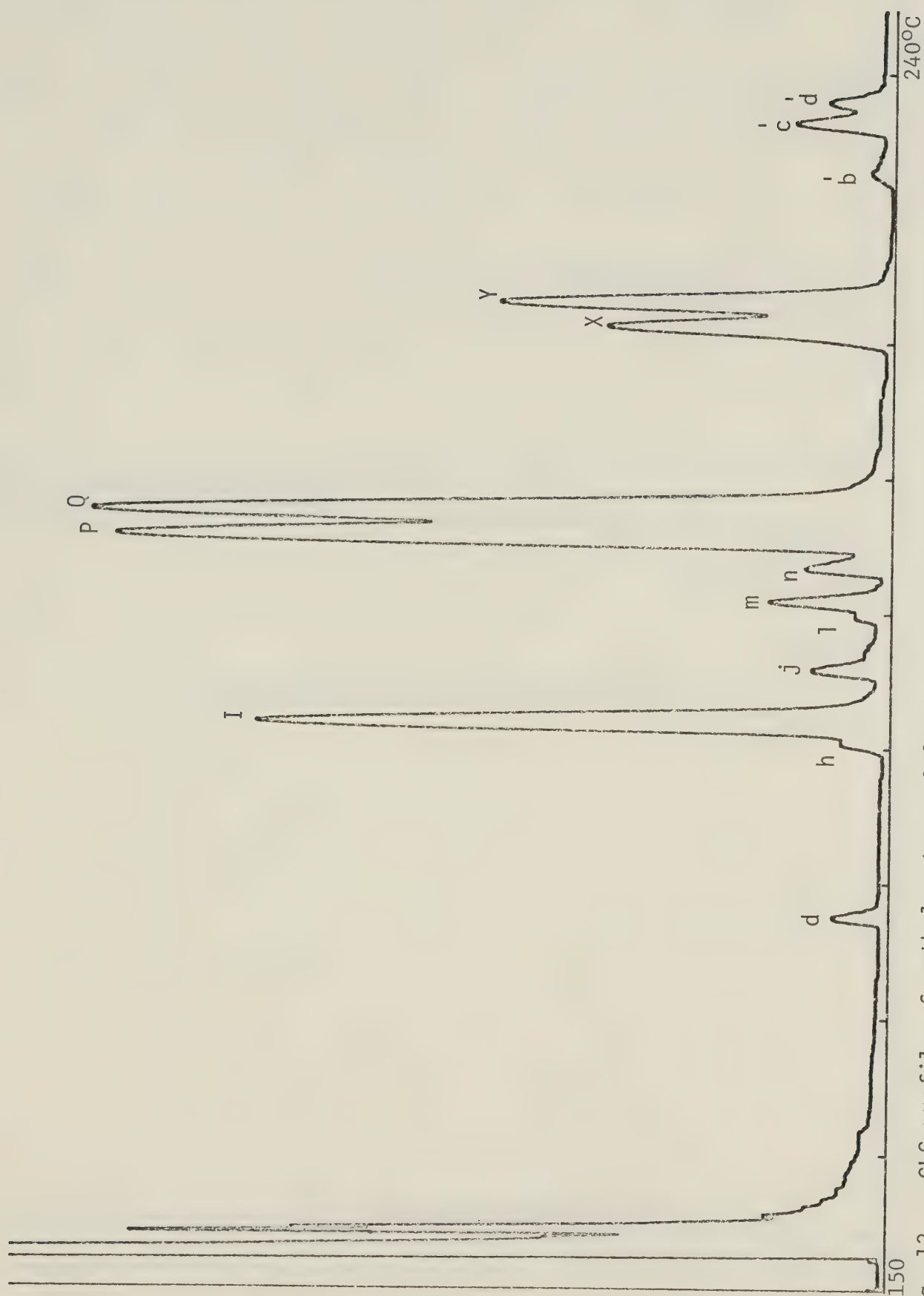


Fig. 13. GLC profile of methyl esters of fatty acids from *Pasteurella multocida* A (10456-56). Letters on peaks refer to identity of acids as shown in Table IX; lower case letters represent trace amounts.

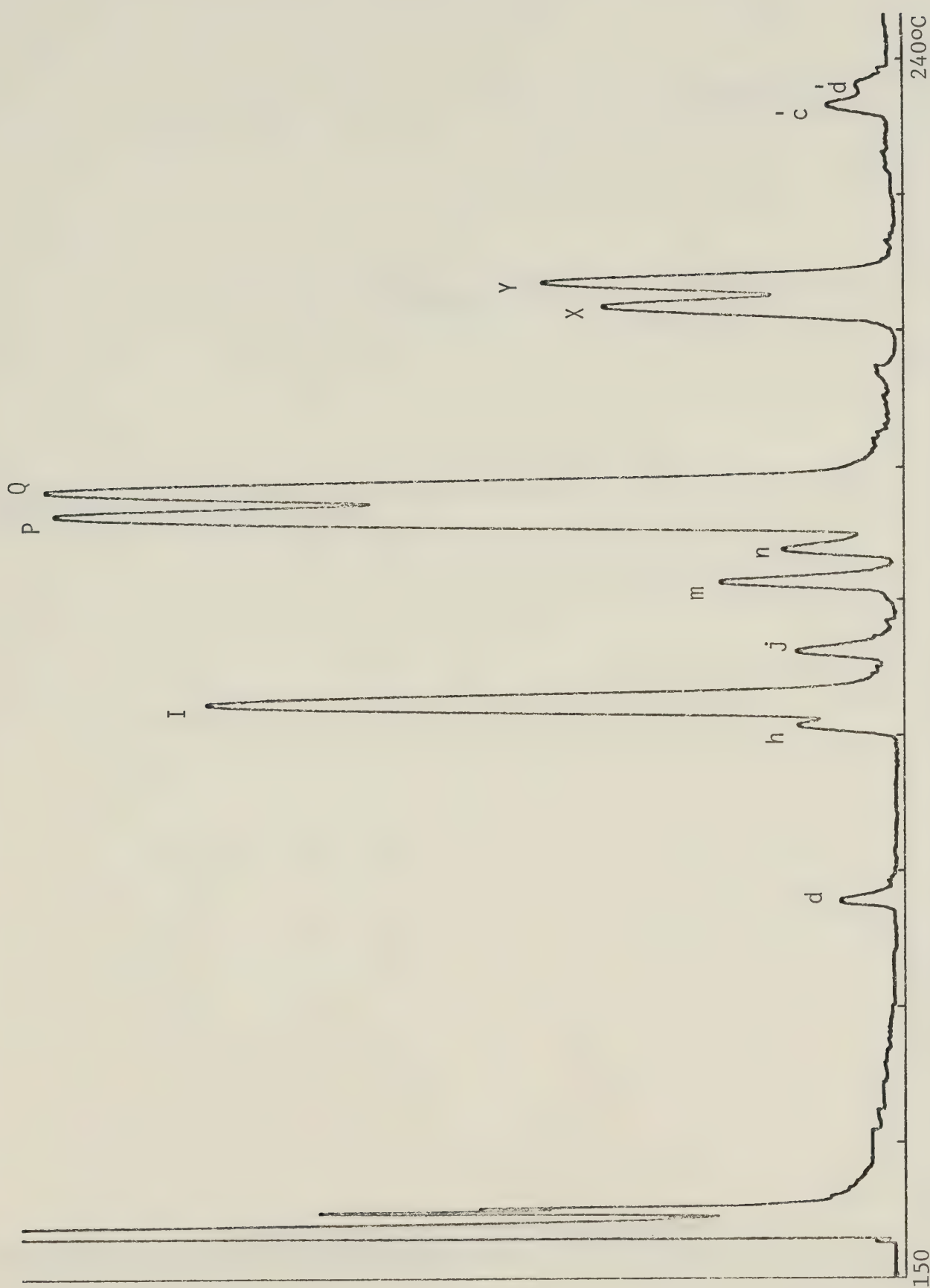


Fig. 14. GLC profile of methyl esters of fatty acids from *Pasteurella multocida* C. Letters on peaks refer to identity of acids as shown in Table IX; lower case letters represent trace amounts.

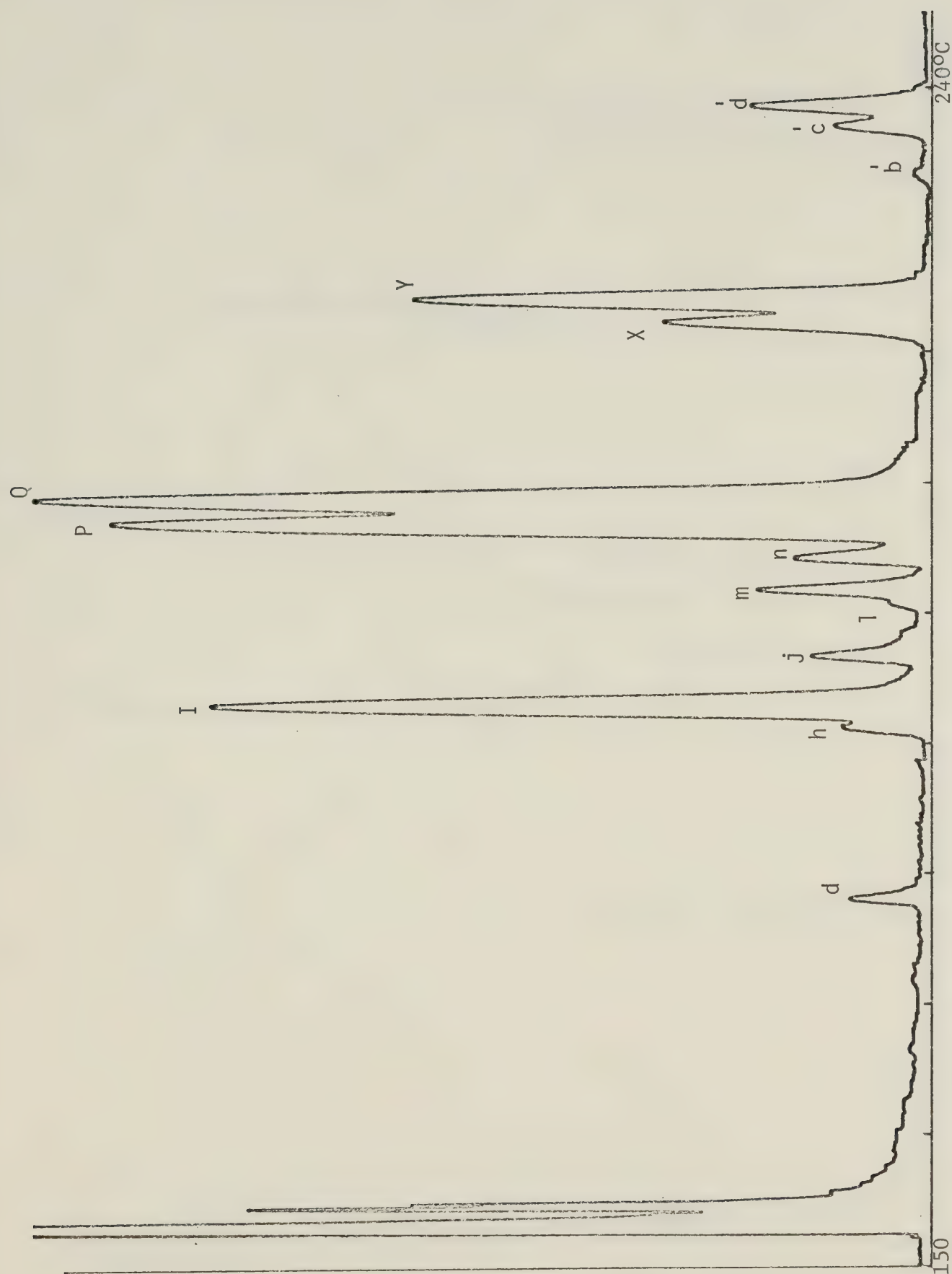


Fig. 15. GLC profile of methyl esters of fatty acids from *Pasteurella multocida* D (10955-55). Letters on peaks refer to identity of acids as shown in Table IX; lower case letters represent trace amounts.

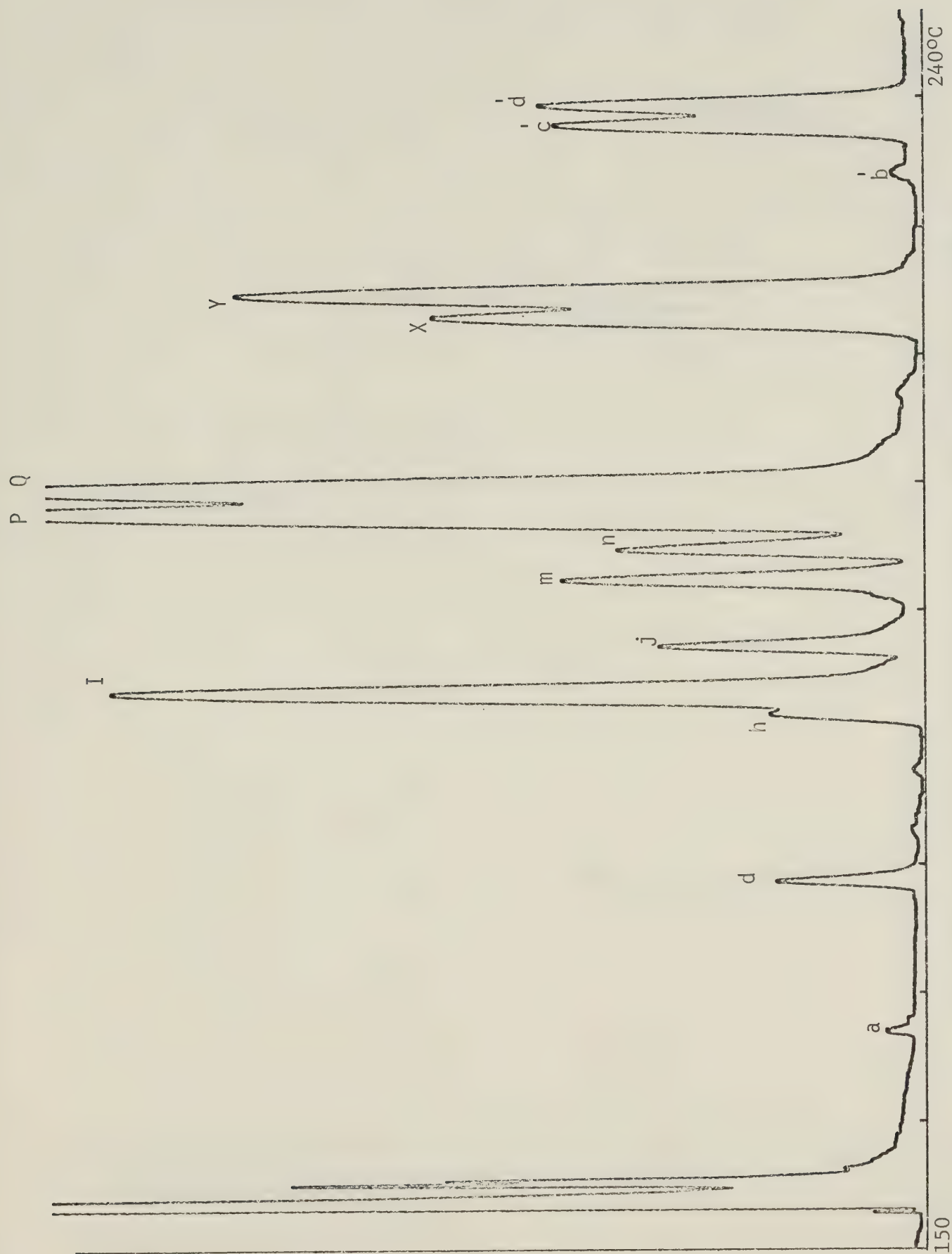


Fig. 16. GLC profile of methyl esters of fatty acids from *Pasteurella multocida* NT (MU 3004-58). Letters on peaks refer to identity of acids as shown in Table IX; lower case letters represent trace amounts.

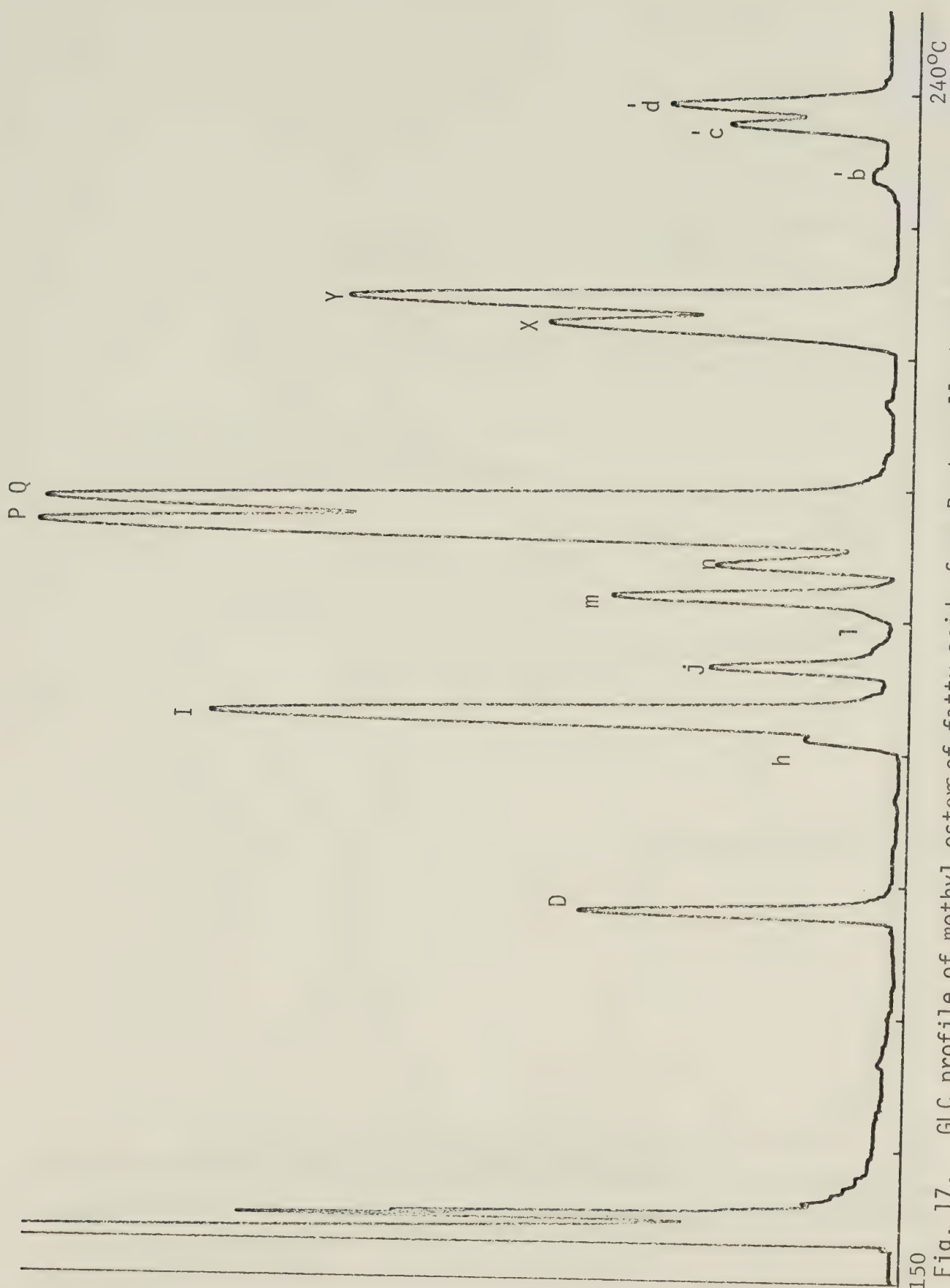


Fig. 17. GLC profile of methyl esters of fatty acids from *Pasteurella haemolytica*. Letters on peaks refer to identity of acids as shown in Table IX; Lower case letters represent trace amounts.

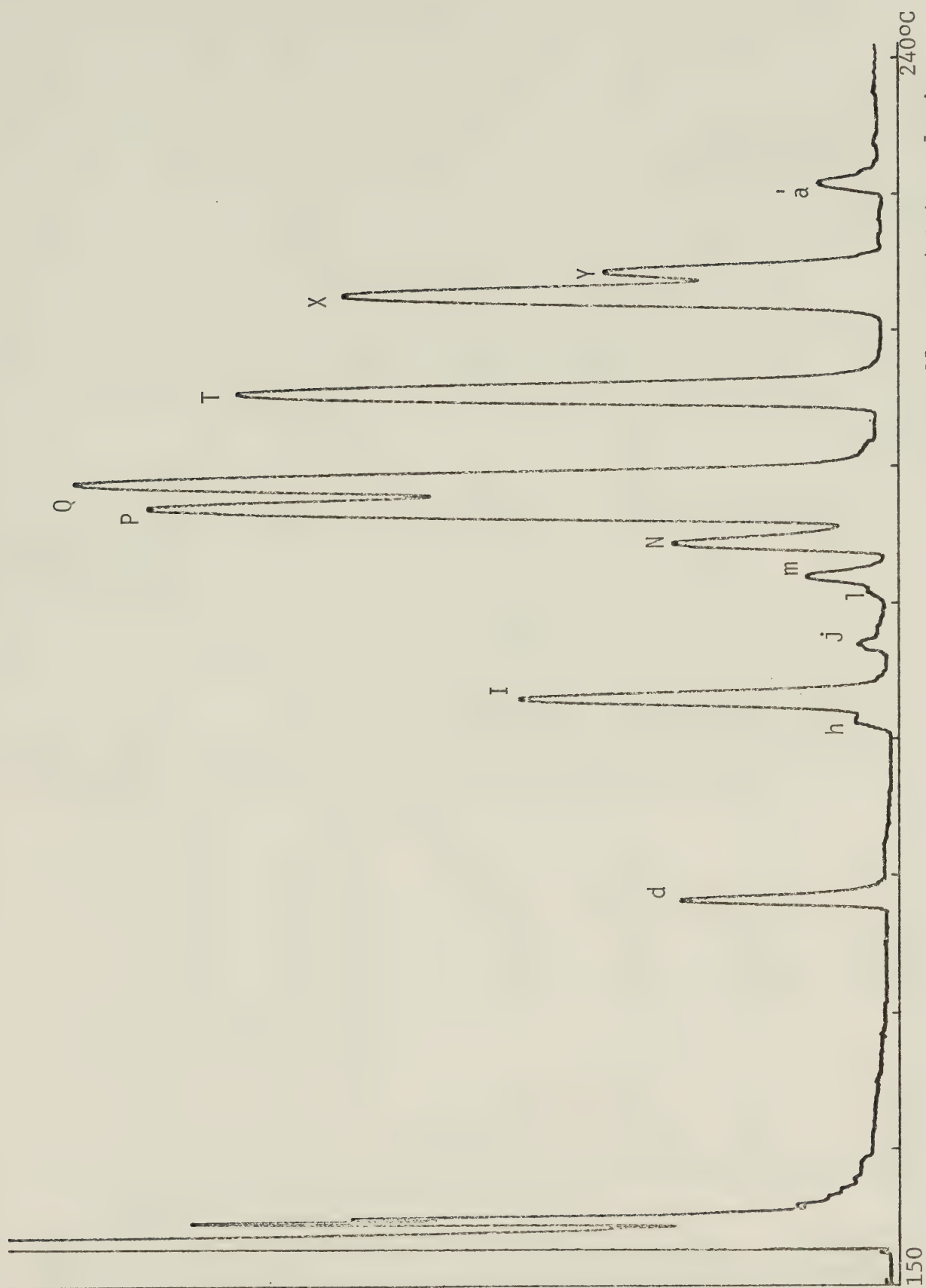


Fig. 18. GLC profile of methyl esters of fatty acids from *Pasteurella pseudotuberculosis*. Letters on peaks refer to identity of acids as shown in Table IX; lower case letters represent trace amounts.

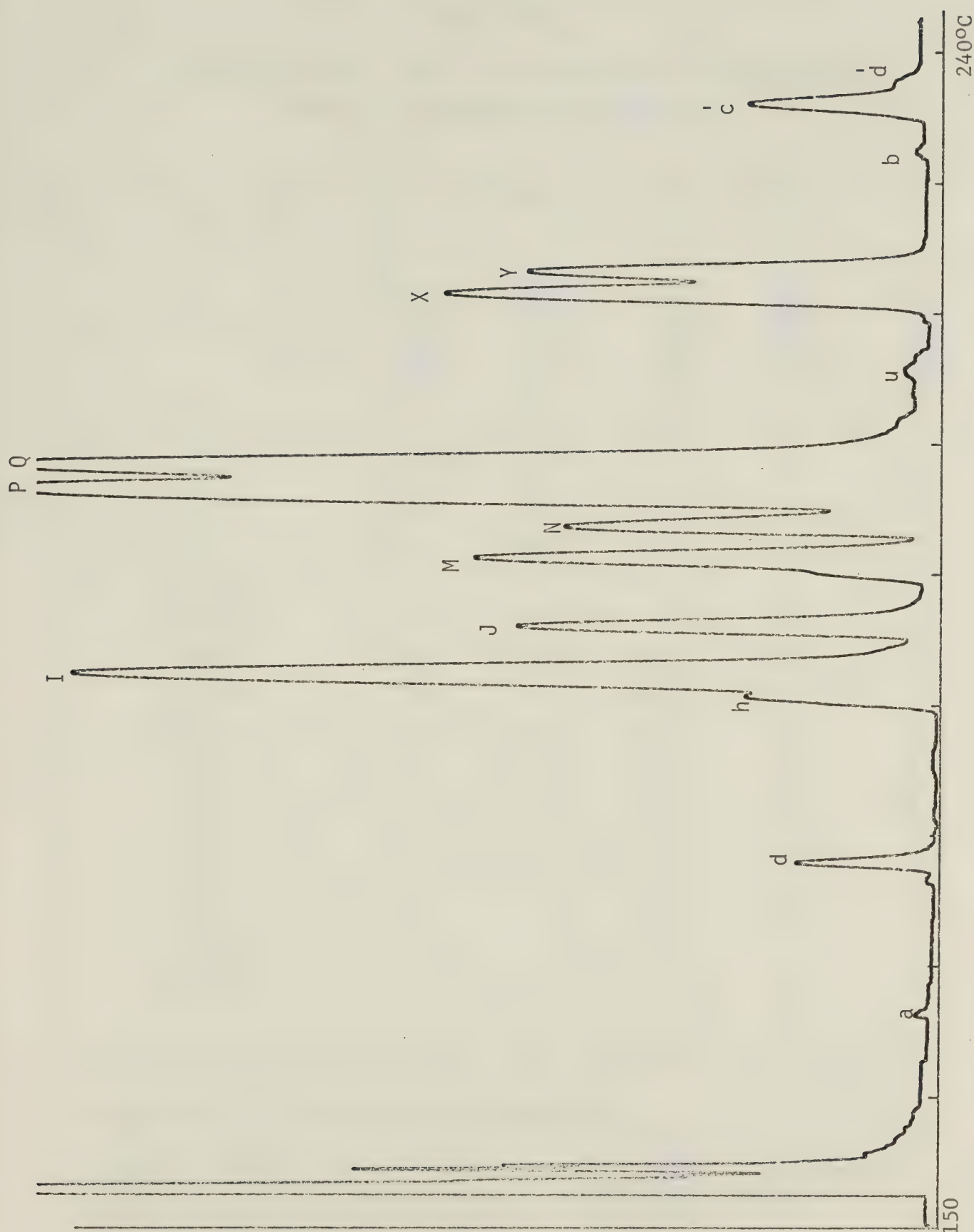


Fig. 19. GLC profile of methyl esters of fatty acids from *Haemophilus aphrophilus* NCTC 5886
 Letters on peaks refer to identity of acids as shown in Table IX; lower case
 letters represent trace amounts.

TABLE IX
COMPARISON OF THE FATTY ACID COMPOSITION OF PASTEURELLA
SPECIES AND HAEMOPHILUS APHROPHILUS

Peak ^a	Fatty acid ^b	Percentage of fatty acids				
		$\frac{\text{P. multocida A}}{10456-56}$	$\frac{\text{P. multocida A}}{5568-61}$	$\frac{\text{P. multocida D}}{675-59}$	$\frac{\text{P. multocida D}}{10955-55}$	$\frac{\text{P. multocida NT}}{5228-58}$
D	12:0	tr ^c	tr	tr	tr	tr
H	(13.71)	tr	tr	tr	tr	tr
I	14:0	15.3	12.4	9.5	15.6	14.9
J	(14.50)	tr	tr	tr	tr	tr
K	(14.72)	tr	-	-	-	-
L	15:0	tr	tr	tr	tr	tr
M	(15.14)	tr	tr	tr	tr	1.0
N	(15.43)	tr	tr	tr	tr	tr
P	16:1	36.8	40.7	38.5	32.5	37.4
Q	16:0	40.9	41.8	43.4	44.9	35.4
X	18:1	2.2	1.5	1.9	tr	3.1
Y	18:0	3.4	2.5	5.2	4.8	5.0
B	(19.24)	tr	-	-	-	-
C	(19.85)	tr	tr	tr	tr	tr
D	-	tr	tr	tr	tr	tr

^a Peak letter as indicated on chromatograms.

^b Number to left of the colon refers to the number of carbon atoms; number to right refers to number of double bonds; numbers in brackets refer to ECL of unidentified fatty acids.

^c tr = less than 1%

Contd. ...

TABLE IX (Contd.)

COMPARISON OF THE FATTY ACID COMPOSITION OF PASTEURELLA
SPECIES AND HAEMOPHILUS APHROPHILUS

Peak ^a	Fatty acid ^b	Percentage of fatty acids					
		<u>P.multocida</u> <u>MU3004-58 NT</u>	<u>P.multocida</u> <u>M 191-60 NT</u>	<u>P.multocida</u> <u>Type C</u>	<u>P.haemolytica</u>	<u>P.pseudotuber-</u> <u>culosis</u>	<u>H.aphrophilus</u>
A	-	-	-	-	-	-	-
D	12:0	tr ^c	tr	tr	1.0	tr	tr
H	(13.71)	tr	tr	tr	tr	tr	tr
I	14:0	13.2	16.2	14.8	13.6	2.4	18.0
J	(14.50)	tr	tr	tr	tr	tr	1.4
L	15:0	tr	tr	tr	tr	tr	tr
M	(15.14)	tr	1.0	tr	tr	tr	2.0 ^d
N	(15.43)	tr	tr	tr	tr	1.0	1.4
P	16:1	37.0	34.2	40.0	38.8	28.8	38.6
Q	16:0	37.0	33.9	37.0	36.2	41.8	33.1
T	(16.83)	-	-	-	-	-	-
U	(17:0	-	-	-	-	-	tr
X	18:1	2.6	2.2	2.3	1.6	9.6	3.1
Y	18:0	7.0	8.2	3.6	6.3	1.2	1.6
A	(18.84)	-	-	-	-	tr	-
C	(19.24)	tr	1.0	tr	tr	-	tr
D	-	tr	tr	tr	tr	-	tr

^a Peak letter as indicated on chromatograms

^b Number to left of the colon refers to the number of carbon atoms; number to the right refers to number of double bonds; numbers in brackets refer to ECL of unidentified fatty acids.

^c tr = less than 1%

^d Incompletely resolved; M was the major contributing peak.

acids in that order, whereas in the non-typable serotypes P (palmitoleic) acid was present in slightly greater proportions than Q (palmitic) acid. P. haemolytica resembled this group in the latter respect. P. pseudotuberculosis was easily separated from the other strains in this group by the presence of T (ECL = 16.83) acid with a concomitant decrease in the percentage of P (palmitoleic) acid. This fatty acid was unique to P. pseudotuberculosis. The concentration of X (octadecenoic) acid was also increased over that present in the other species of Pasteurella.

H. aphrophilus NCTC 5886 was placed in Group IV on the basis of qualitative and quantitative similarities (Fig. 19 and Table IX). This organism was at one time considered related to B. corrodens.

Signatures of the bacterial strains under study

An overall comparison of the fatty acid composition of the strains used in this study is illustrated in Table X, where a signature or fingerprint for each strain was established as described by Henis et al. (22). Gross distinctions can be made between each group by considering the presence or absence of letters representing major fatty acids. Differentiation between strains is not always possible within a group; however, this could be made possible by application of statistical analysis (22) to determine whether the concentrations of the fatty acids of a strain are significantly different from each other. The application of

TABLE X
SIGNATURES OF BACTERIA INCLUDED IN THIS STUDY BASED ON
CELLULAR FATTY ACID COMPOSITION

Group	Organism	Signature
I	<u>B. corrodens</u> NCTC10956 (333/54-55)	QXPDIY-ghkl ['] tub ['] c
	<u>B. corrodens</u> NCTC A40/68	QXPDIY-ghkl ['] tub ['] c
	B-916	QXPDIY-ghkl ['] tub ['] c
	S-209	QXPDIY-ghkl ['] tub ['] c
	53P	XQPDYI-aghkl ['] tub ['] c
	53W	XQPDYI-ghkl ['] tub ['] c
II	B-912	XQDIY(ML)-afjnp ^{''} uabc ^{''}
	4053	XQYID-afj(lm)np ^{''} uabc ^{''}
	EDMH-1	XQYID-afj(lm)np ^{''} uabc ^{''}
	4282 (Lane)	XQYIDP-afj(lm)nub ^{''} c
III	3936	KEQV-himopr ['] suwyxab [']
	4482	KQVEY-hiop ['] rszwab [']
	<u>B. fragilis</u> NCTC 9343	KQLIYVEH-cdfopsuwz ['] b
	<u>B. oralis</u>	KQLYSV-defhiopuwz
	<u>B. melaninogenicus</u>	KHEOIQS-abcdfluvwy ['] bc [']

^a Peaks arranged in decreasing order of magnitude; lower case letters represent trace amounts (less than 1%) and are listed in alphabetical order; underlined letters signify that the fatty acids represented by these letters were present in equal amounts.

^b Peaks represented by (ML) or (lm) were not completely resolved from one another; M or m was the major contributing peak.

TABLE X (Contd.)

SIGNATURES OF BACTERIA INCLUDED IN THIS STUDY BASED ON
CELLULAR FATTY ACID COMPOSITION

Group	Organism	Signature
IV	<u>P. multocida</u> A (10456-56)	QPIYX-dhjk ['] lmn ['] bcd [']
	<u>P. multocida</u> A (5568-61)	QPIYX-dhj ['] lmncd [']
	<u>P. multocida</u> D (675-59)	QPIYX-dhj ['] lmncd [']
	<u>P. multocida</u> D (10955-55)	QPIY-dhj ['] lmncd [']
	<u>P. multocida</u> NT (5228-58)	PQIYXM-dhj ['] lncd [']
	<u>P. multocida</u> NT (MU3004-58)	<u>PQIYX</u> -dhj ['] lmncd [']
	<u>P. multocida</u> NT (M191-60)	PQIYXMC ['] -dhjnd [']
	<u>P. multocida</u> C	PQIYX-dhj ['] lmncd [']
	<u>P. haemolytica</u>	PQIYXD-hj ['] lmncd [']
	<u>P. pseudotuberculosis</u>	QPTXIYN-dhj ['] lma [']
	<u>H. aphrophilus</u> NCTC 5886	PQIXMJN-adhu ['] cd [']

a Peaks arranged in decreasing order of magnitude; lower case letters represent trace amounts (less than 1%) and are listed in alphabetical order; underlined letters signify that the fatty acids represented by these letters were present in equal amounts.

b Peaks represented by (ML) or (lm) were not completely resolved from one another; M or m was the major contributing peak.

statistical analysis was not justified in this study since only two replicates of each strain were analyzed.

Effect of age of culture, oxygenation and growth medium on fatty acid composition

Fatty acid composition has been shown to be influenced by the age of the culture (42). Experiments were carried out to determine whether age effects would modify the grouping of the strains being investigated. The fatty acid composition of the anaerobic corroding strain 4053 was compared after three and four days of growth on Wahren and Holme's modified medium. Visual examination of the fatty acid methyl ester profile indicated no qualitative or major quantitative changes. Comparison of the percentages of the principal fatty acid as shown in Table XI indicates very little change in the relative proportions of the fatty acids at three or four days of growth. Due to the fact that this organism had to be cultured on a solid medium, and not knowing whether valid conclusions could be made from the above, another experiment was performed with Pasteurella multocida Type C, which grows very well in the liquid form of the same medium. The culture was grown on a shaker at 37°C; half of the culture was harvested during logarithmic growth ($O.D._{550} = 1.2$) and the other half after 5 hours of stationary growth ($O.D._{550} = 1.7$). From Table XI it is possible to conclude that no qualitative or major quantitative changes occurred. There was a slight increase of Q

TABLE XI

EFFECT OF AGE OF CULTURE ON FATTY ACID COMPOSITION OF
PASTEURELLA MULTOCIDA C AND CORRODING STRAIN 4053

Peak ^a	Fatty acid ^b	Percentage of fatty acids			
		<u>P. multocida</u> C		4053	
		logarithmic OD ₅₅₀ = 1.2	stationary OD ₅₅₀ = 1.7	3 days	4 days
A	-	-	-	tr ^c	-
D	12:0	tr	tr	3.3	3.4
F	-	-	-	tr	-
H	(13.71)	tr	tr	-	-
I	14:0	16.1	16.8	4.4	3.1
J	(14.50)	tr	tr	tr	tr
L	15:0	-	-	tr	tr
M	(15.14)	tr	tr	tr	tr ^d
N	(15.43)	tr	tr	tr	tr
P	16:1	42.2	39.8	tr	tr
Q	16:0	35.8	38.2	9.1	7.6
U	17:0	-	-	tr	tr
X	18:1	1.8	1.4	72.2	77.8
Y	18:0	2.6	2.0	8.2	6.6
A	(18.84)	-	-	tr	tr
B	(19.24)	-	-	tr	tr
C	(19.85)	tr	tr	tr	tr
D	-	tr	tr	-	-

^a Peak letter as indicated on chromatograms.

^b Number to left of the colon refers to the number of carbon atoms; number to right refers to number of double bonds; numbers in brackets refer to ECL of unidentified fatty acids.

^c tr = less than 1%

^d Incompletely resolved; M was the major contributing peak.

(palmitic) acid at the expense of P (palmitoleic) acid in the stationary phase; however, this may be due to experimental variation only. Perhaps if the first half of the culture had been harvested in early logarithmic growth, greater differences might have been noted.

It has been reported that an increase in oxygenation produces an increase in the amount of unsaturated fatty acids (30,70). P. multocida Type C was grown both aerobically and anaerobically on Wahren and Holme's modified agar. From Table XII it is apparent that these growth conditions had little effect on the percentage composition of the fatty acids of P. multocida Type C. Kaneda (30) postulated that the rate of diffusion of oxygen in solid medium is slower than in liquid medium, hence this may explain why no obvious changes in the concentration of unsaturated fatty acids (palmitoleic and octadecenoic) at the expense of their saturated homologs (palmitic and stearic) were observed. On the other hand, if a comparison is made between the results of P. multocida Type C cultures grown aerobically on a solid medium (Table XII) and in a liquid medium (Table XI) no changes beyond those expected from experimental variations were observed.

Another factor which has been shown to influence fatty acid composition is the growth medium (17, 42, 50). This factor was tested in an experiment in which Bacteroides fragilis NCTC 9343 was cultured on two types of solid medium: Wahren and Holme's modified medium and 2.5% Nutrient Broth No. 2 (Difco) supplemented with 5%

TABLE XII

EFFECT OF ANAEROBIC AND AEROBIC GROWTH CONDITIONS ON
THE FATTY ACIDS OF PASTEURELLA MULTOCIDA C

Peak ^a	Fatty acid ^b	Percentage of fatty acids	
		Anaerobic	Aerobic
D	12:0	tr	tr
H	(13.71)	tr	tr
I	14:0	14.8	14.4
J	(14.50)	tr	tr
L	15:0	tr	tr ^d
M	(15.14)	tr	tr
N	(15.43)	tr	tr
P	16:1	40.4	42.4
Q	16:0	37.0	37.4
X	18:1	2.3	1.8
Y	18:0	3.6	2.2
ⁱ C	(19.85)	tr	tr
ⁱ D	-	tr	tr

^a Peak letter as indicated on chromatograms

^b Number to left of colon refers to number of carbon atoms; number to right refers to number of double bonds; numbers in brackets refer to ECL of unidentified fatty acids.

^c tr = less than 1%

^d Incompletely resolved; M was the major contributing peak.

sheep blood. The results (Table XIII) show that the relative amounts of the principal fatty acids were not altered to any extent. Both media are classified as complete media; if this experiment were to be repeated with a minimal medium it is more than likely that quantitative differences would be observed.

TABLE XIII
EFFECT OF GROWTH MEDIUM ON THE FATTY ACID COMPOSITION
OF B. FRAGILIS NCTC 9343

Peak ^a	Fatty acid ^b	Percentage of fatty acids	
		Wahren and Holme's modified medium	Blood agar medium
C	-	tr ^c	-
D	12:0	tr	-
E	(12.60)	1.3	tr
F	-	tr	tr
H	(13:71)	1.0	tr
I	14:0	2.1	1.2
K	(14.72)	70.0	70.2
L	15:0	9.8	8.0
O	(15.64)	tr	tr
P	16:1	tr	tr
Q	16:0	10.8	12.0
R	-	tr	tr
S	(16.71)	tr	tr
U	17:0	tr	tr
V	(17.48)	1.5	1.6
W	(17.74)	tr	2.6
Y	18:0	2.1	1.6
Z	(18.52)	tr	tr
B	(19.24)	tr	tr

^a Peak letter as indicated on chromatograms.

^b Number to left of the colon refers to the number of carbon atoms; number to right refers to number of double bonds; numbers in brackets refer to ECL of unidentified fatty acids.

^c tr = less than 1%

III

CLASSIFICATION ON THE BASIS OF METABOLIC END-PRODUCTS

METHODS AND MATERIALS

Growth medium

A further modification of Wahren and Holme's (72) medium was necessary due to the relatively large proportion of certain peaks in the ether extract of the medium. GLC analysis of each medium component was carried out and it was concluded that tryptone (Difco) was the major source of these peaks. Therefore 1% peptone was substituted for 1.5% tryptone; even then, different batches of peptone contributed varying amounts of peaks to the complete medium. A batch which contributed the least to the peaks encountered was selected.

Other media were tried such as the modified Proom and Knight's medium used by Henis et al. (22) supplemented with haemin (Eastman Organic Chemicals) at a final concentration of 5 µg/ml. This medium had fewer peaks but did not support the growth of the more fastidious organisms under study very well.

Growth conditions

The bacteria were grown on modified Wahren and Holme agar in Baird and Tatlock cold catalyst anaerobic jars evacuated and filled with 90% H₂ and 10% CO₂. The cultures were incubated at 37°C for three to four days, after which the cells were washed from the agar with phosphate buffered saline and the turbidity of the suspension was adjusted to an absorbancy of 0.50 at 550 mµ.

To nine acid-washed screw-capped bottles, each containing 10 ml of medium, was added 0.2 ml of the inoculum. The cultures were incubated anaerobically, with the caps screwed loosely as previously described, for 3, 6, and 10 days (three replicates for each).

Extraction procedure

The extraction procedure is essentially that described by Brooks and Moore (8). Prior to extraction a loopful from each culture was subcultured onto modified Wahren and Holme's and blood agar plates to check the purity of the culture. The cultures were centrifuged at 2500 rpm for 45 minutes at 4°C, the supernatant removed and acidified with 50% (v/v) aqueous H_2SO_4 to about pH 2. Acidified cultures were extracted with 3 ml of anhydrous ethyl ether (Fisher Scientific) by inverting the glass-stoppered centrifuge tubes 20 times, then centrifuged briefly to break the ether-water emulsion. The ether layer was transferred to a 3 ml teflon capped glass vial and stored at -20°C until analyzed. Prior to analysis, the acid ether extract was concentrated to approximately 0.05 to 0.1 ml by a moderate stream of nitrogen led into the vial through a fine point Pasteur pipette in a fume hood. Samples were analyzed either immediately or within 24 hours after collection, during which time no chemical changes were observed. Two replicates from each strain and from each incubation period were extracted.

All glassware used was cleaned with dichromic acid and thoroughly rinsed with distilled water. Control runs were performed on the ether-acid mixture.

GLC analysis of fermentation patterns

The bacterial metabolites were analyzed by use of a Carlo Erba Fractovap Model GV dual column gas chromatograph (Carlo Erba, Milan, Italy) equipped with a hydrogen-flame ionization detector. The output was recorded on a Honeywell strip chart recorder and integrated on an Infotronics CR 100 digital integrator.

Separation was effected on a 3m column [10% 20 M Carbowax on Chromosorb W, 60/80 mesh (Chromatographic Specialties, Brockville, Ontario), packed in 6 mm outer diameter stainless steel tubing]. Operating parameters of the instrument were: injection temperature 180°C; detector temperature 190°C, column temperature was linearly programmed from 50°C to 180°C; carrier gas, nitrogen at 50 ml per minute; hydrogen adjusted to give maximum sensitivity (30 ml/minute) air; 300 ml per minute; input and output attenuation, 10^2 and 8 respectively; chart speed, one-half inch per minute.

For routine analysis 8 to 10 μ l of the concentrated ether extract was analyzed for 31 minutes after injection under the above-stated conditions. This time interval was sufficient to detect straight chain alcohols of 1 to 12 carbon atoms in length and fatty acids of 1 to 5 carbons in length (Figs. 20 and 21).

1.	2 methyl-2-propanol	6.	2 methyl-1-propanol	11.	1-pentanol	15.	1-octanol
2.	methanol, 2-propanol	7.	2-pentanol	12.	2-methyl-1-butanol 2-methyl-1-pentanol	16.	1-nonanol
3.	ethanol	8.	1-butanol	13.	1-hexanol	17.	1-decanol
4.	1-propanol	9.	4-methyl-2-pentanol	14.	1-heptanol	18.	1-undecanol
5.	2-butanol	10.	2-methyl-1-butanol			19.	1-dodecanol

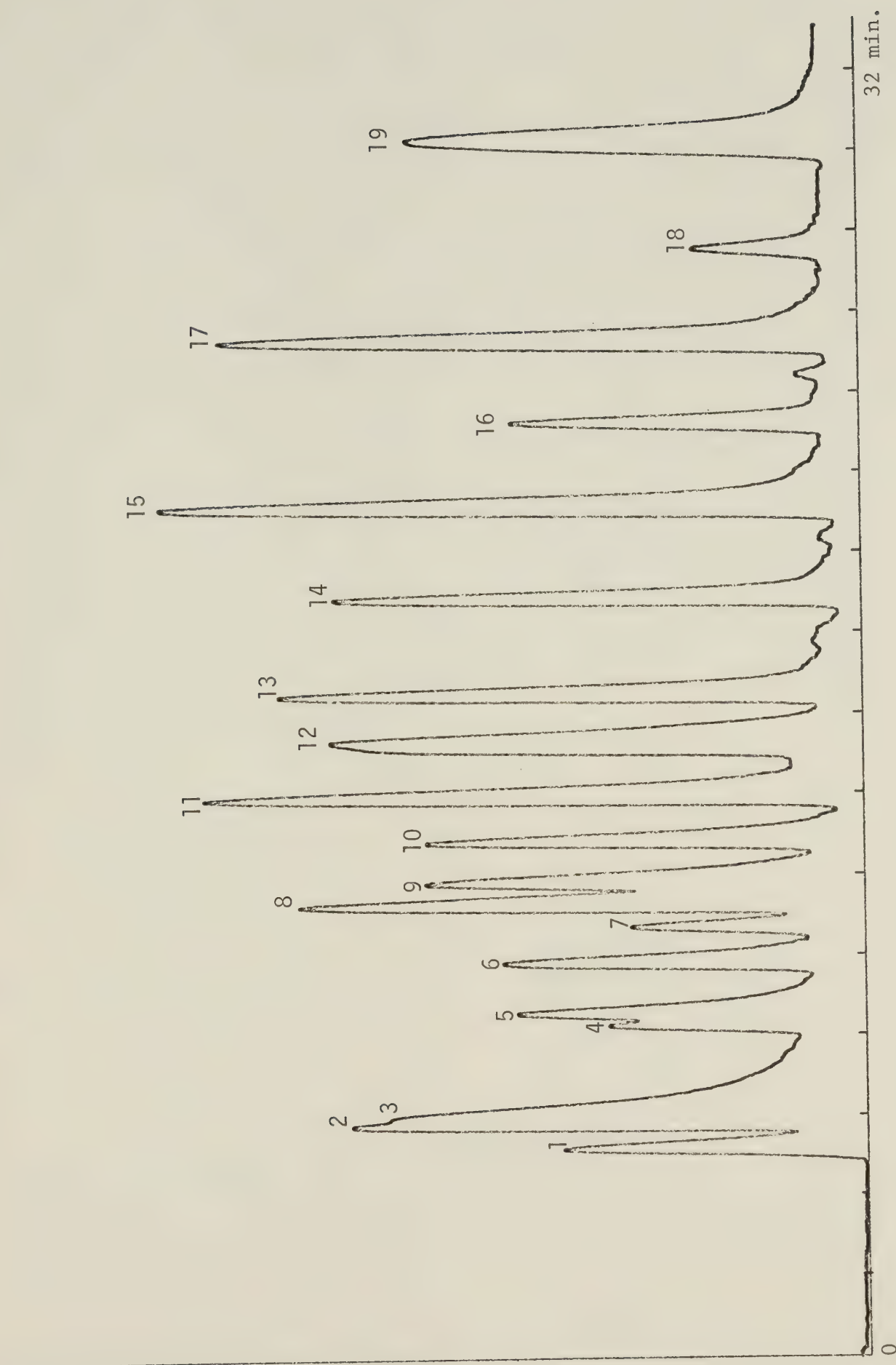


Fig. 20. GLC profile of alcohol standards.

- | | | | |
|----|------------|----|------------|
| 1. | acetic | 4. | isovaleric |
| 2. | isobutyric | 5. | valeric |
| 3. | butyric | | |

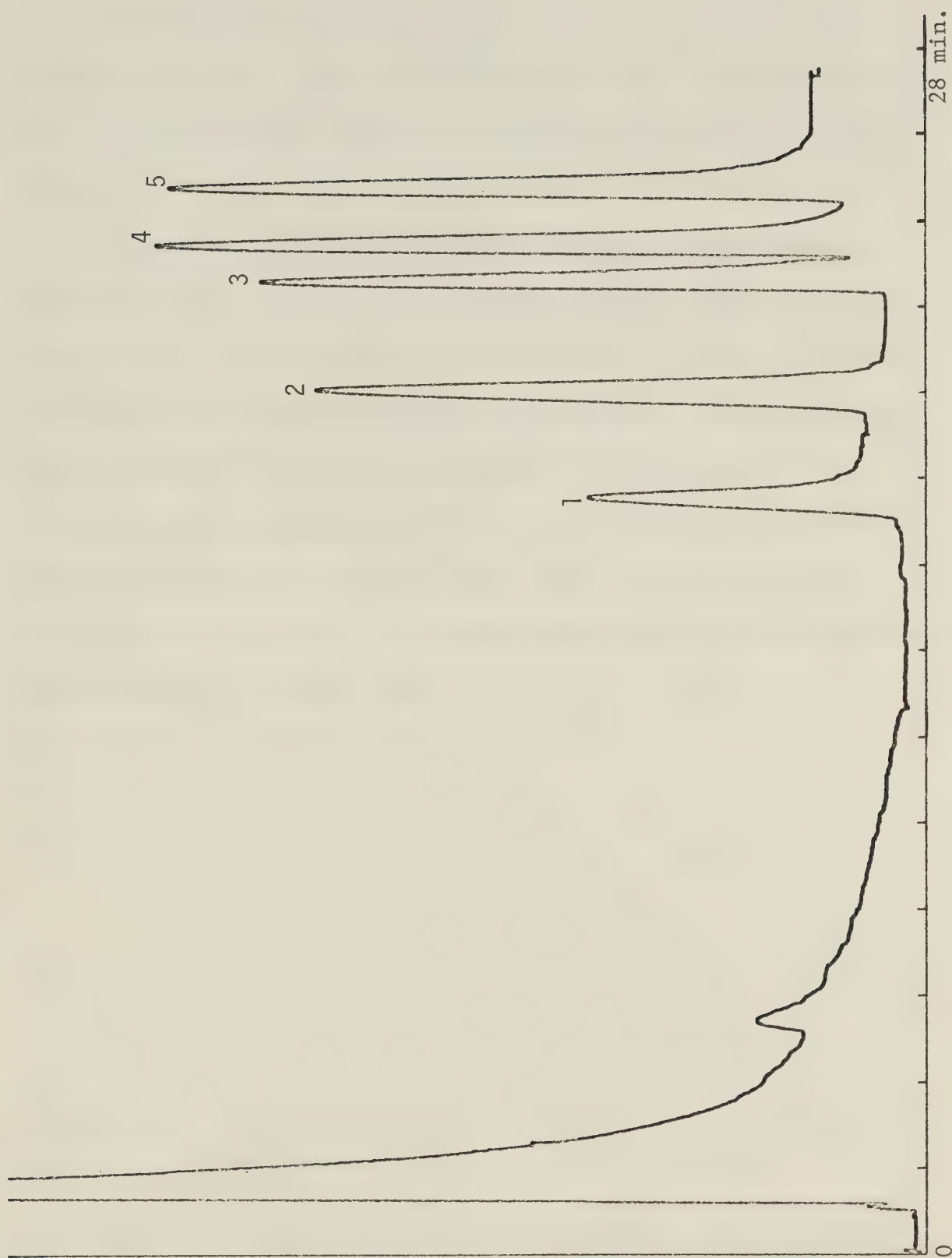


Fig. 21. GLC profile of short chain fatty acid standards.

For purposes of illustration, 8 to 10 μ l of sample were analyzed under the same conditions except that a logarithmic scale was used and the chart speed was reduced to one-quarter inch per minute.

Tentative identification was attempted by comparison of retention times with those of standard alcohols and short chain fatty acids. The straight and branched chain alcohol standards were supplied by Applied Science Laboratories. The short chain fatty acids were prepared as described by Cato et al. (11).

The peaks designated as "f" on the chromatograms were tentatively identified as acetic acid; peak "h" was tentatively identified as 1-octanol. All other peaks differed from the standards in their retention time.

RESULTS

The 26 cultures being investigated can be separated into seven distinct fermentation patterns by observing the presence or absence of peaks representing metabolites synthesized by these microorganisms. It was not possible to obtain quantitative data because of trailing of the solvent peak which gave values for the first three peaks which were out of proportion to those of the remaining peaks. Hence, the grouping of strains on the basis of bacterial end-products was based primarily on visual comparison of the various GLC profiles.

With the more fastidious microorganisms, end-products were not detectable before three days of growth. No qualitative changes were noted with increased incubation. Quantitative changes were noted after six days of continued incubation but no further quantitative changes occurred after 10 days.

In Table XIV are listed all the peaks encountered in the GLC profiles according to increasing retention time. The retention times listed are defined only for the GLC conditions under which these experiments were performed, and represent the average retention time of the peaks as determined by all the replicates in which they were detected.

Fig. 22 shows the results of the analysis of uninoculated medium incubated under the same conditions as the test organisms. No qualitative nor apparent quantitative changes in these peaks

TABLE XIV
PEAKS ENCOUNTERED IN THE VARIOUS FERMENTATION PATTERNS
DUE TO MEDIUM AND/OR BACTERIAL METABOLITES

Peak	Retention time (sec.)	Present in medium	Bacterial metabolite ^a
1	346	+	
2	410	+	a
3	702	+	
4	789		b
5	957		c
6	1064		d
7	1149	+	e
8	?		f
9	1213		g
10	1271	+	h
11	1304	+	i
12	1385	+	
13	1439	+	j
14	1529	+	
15	1602	+	
16	1659	+	

^a Peak letter as indicated on chromatograms.

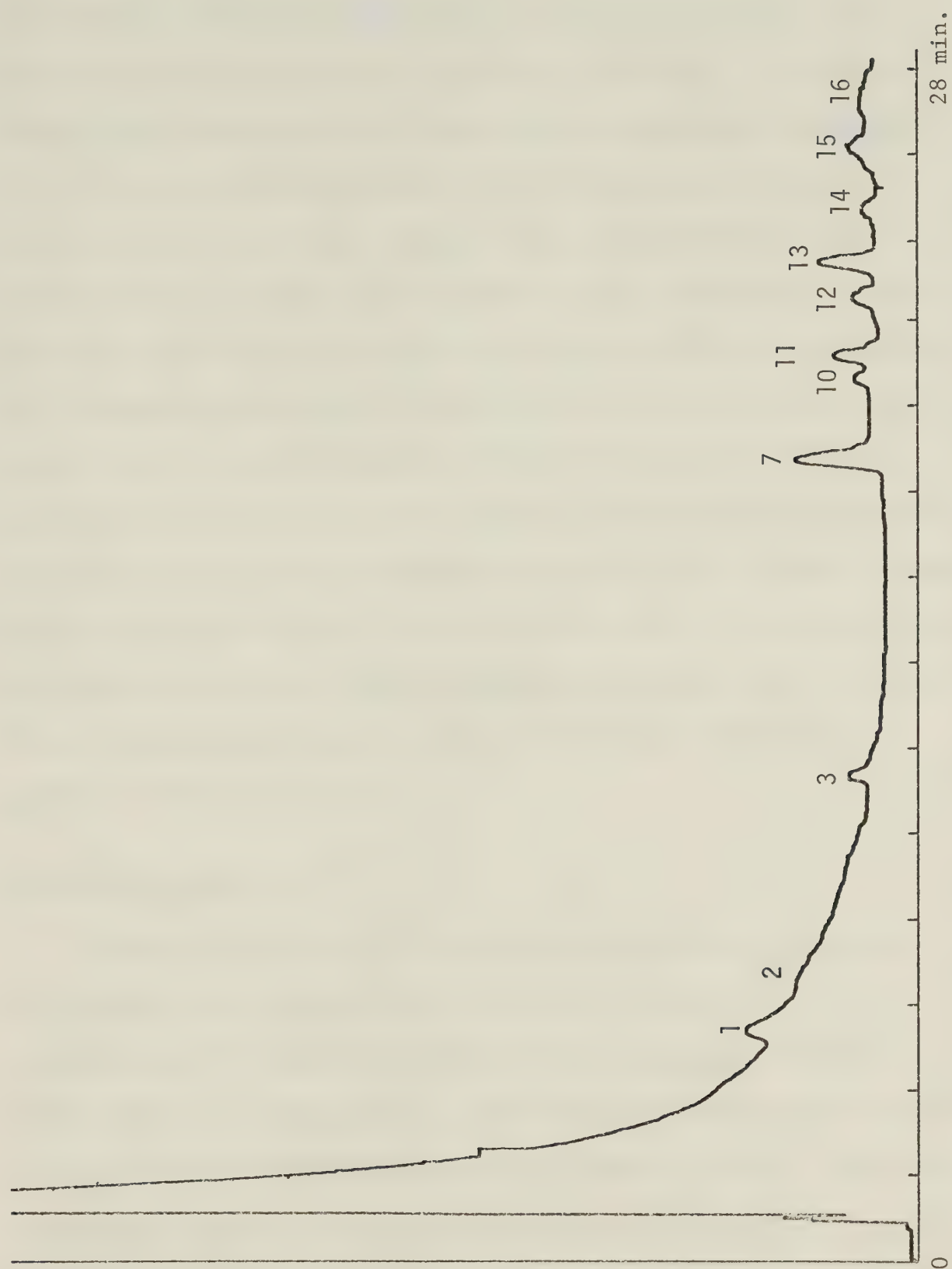


Fig. 22. GLC profile of ether extract from Wahren and Holme's modified liquid medium after 3 days of anaerobic incubation.

were observed following incubation for three or six days. The first small peak is due to an impurity in the ether. To facilitate visual interpretation of the various chromatograms illustrated in this study, only those peaks due to the presence of bacterial metabolites are labelled. Some of these peaks are due to the medium as well as bacterial end-products but a substantial increase in peak height relative to other medium peaks indicates the presence of a bacterial end-product either identical to that present in the medium or of a similar retention time. The end-product peaks were labelled with letters to distinguish them from the medium peaks as well as to establish a signature for each strain in order to permit rapid differentiation as illustrated in Table XV. The peaks making up each signature are listed according to increasing retention time. These signatures must not be confused with those obtained by cellular fatty acid analysis.

Fermentation patterns

Fermentation pattern I revealed no detectable end-products. This pattern was characteristic of the anaerobic strains of corroding bacilli tentatively designated B. *corrodens*: B-912, EDMH-1, 4053, and 4282 (Lane). One chromatogram for B-912 is shown in Fig. 23 and is representative of this group. In this figure the peak heights appear to be greater than those of the medium (Fig. 22) but their relative proportions remain almost identical so that if there are end-products, they are present in concentrations too small

TABLE XV
SIGNATURES OF VARIOUS BACTERIA INCLUDED IN THIS STUDY
BASED ON METABOLIC END-PRODUCTS

Fermentation pattern	Strain	Signature ^a
I	B-912	-
	EDMH-1	-
	4053	-
	4282 (Lane)	-
II	<u>B. corrodens</u> NCTC 10596 (333/54-55)	bde ^b
	<u>B. corrodens</u> NCTC A40/68	bde
	B-916	bde
	S-209	bde
	53P	bde
	53W	bde
III	<u>B. melaninogenicus</u>	deij
IV	<u>B. fragilis</u> NCTC 9343	bcdehij
	<u>B. oralis</u>	bcdehij
V	4482	bcdeghij
	3936	abcde ^b ghij
VI	<u>P. multocida</u> (all serotypes)	ae
	<u>P. haemolytica</u> NCTC 5885	ae
	<u>P. pseudotuberculosis</u>	ae
VII	<u>H. aphrophilus</u> NCTC 5886	de

^a Peaks arranged in order of increasing retention time.

^b Underlined letters represent major end-products.

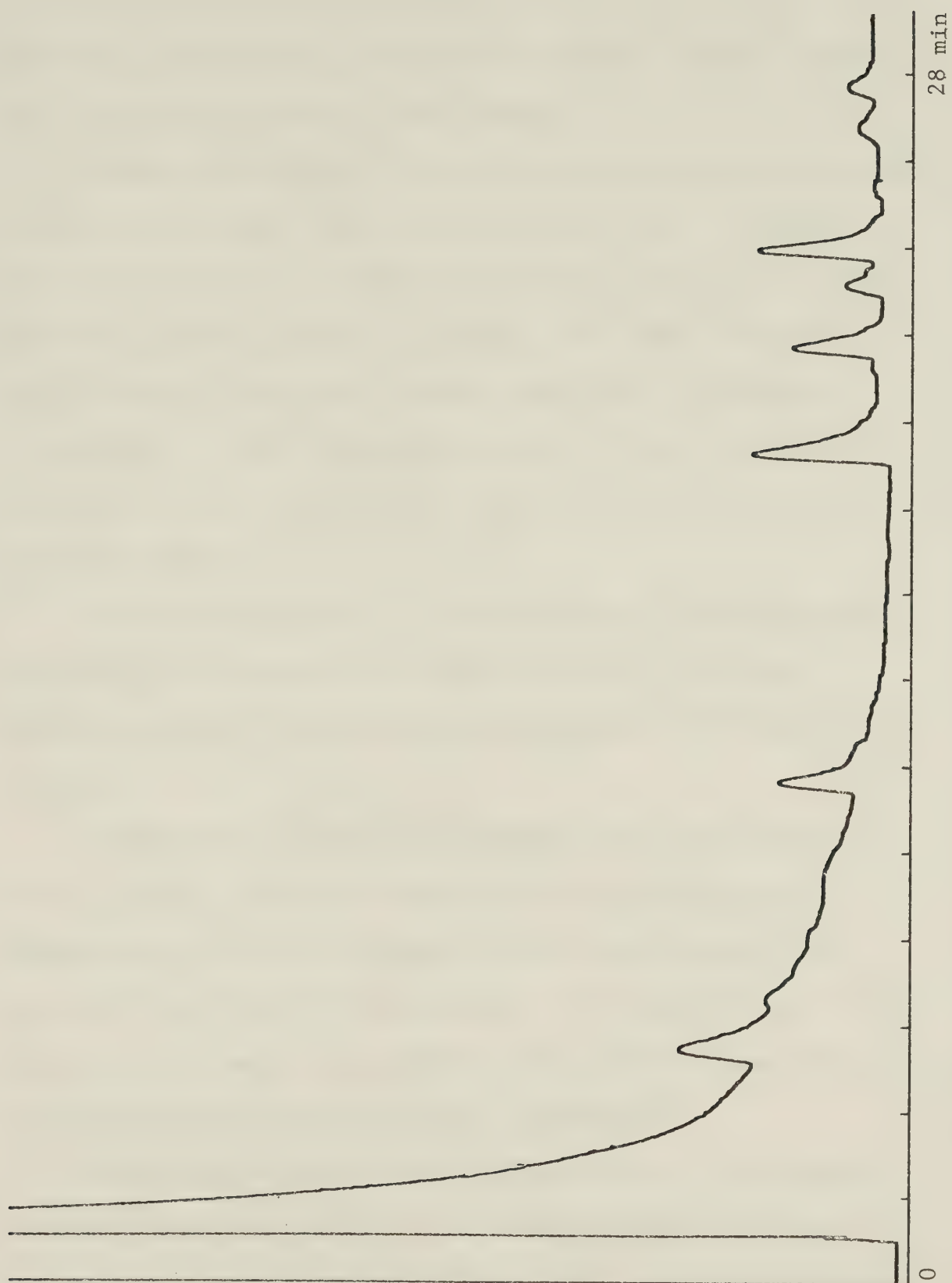


Fig. 23. GLC profile of metabolic end-products in ether extract from corroding strain B-912 representative of fermentation pattern I.

to be detected by visual comparison of these two profiles. The increase in peak heights in the test organisms are most probably due to a more concentrated ether extract.

Fermentation pattern II was characterized by one major end-product and two minor ones, as shown in Fig. 24. This pattern was representative of all the facultatively anaerobic strains of corroding bacilli, namely B. corrodens NCTC 10596 (333/54-55) and NCTC A40/68, and those strains tentatively designated as B. corrodens: B-916, S-209, 53P and 53W. These strains all had similar profiles which permitted them to be exclusively and homogeneously grouped.

Fermentation pattern III was unique to B. melaninogenicus. Three major end-products, as illustrated in Fig. 25, were detectable, plus a fourth end-product present in very low concentration.

Fermentation pattern IV was representative of B. fragilis and B. oralis. These two members of the Bacteroides genus produced qualitatively and quantitatively similar patterns as shown in Figs. 26 and 27 respectively. This pattern was characterized by four major end-products, three of which were shared by B. melaninogenicus but in different proportions.

Fermentation pattern V was characteristic of two anaerobic strains of corroding bacilli also tentatively designated as B. corrodens, namely strains 4482 and 3936. Profiles of these two organisms are shown in Figs. 28 and 29 respectively. These

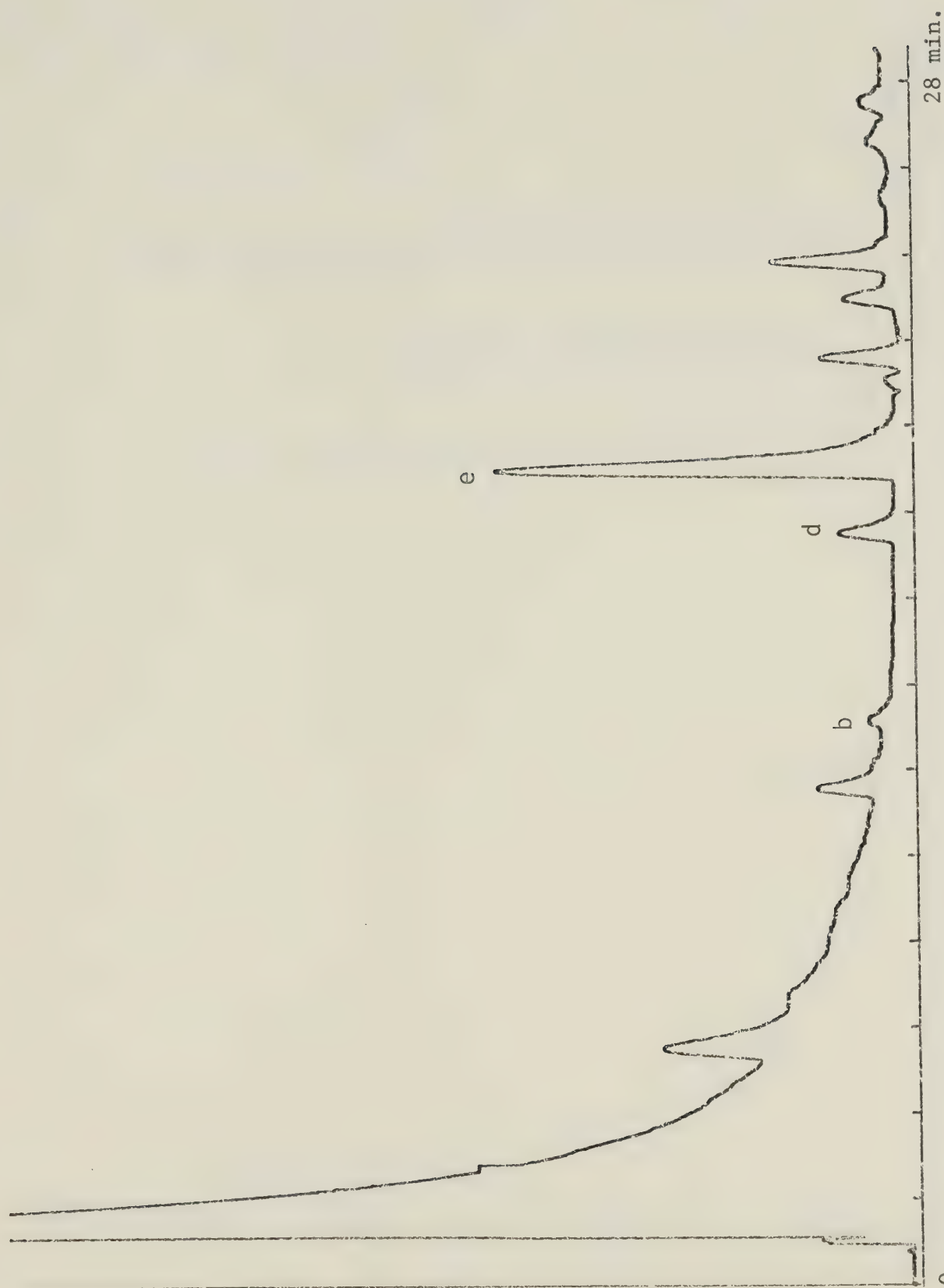


Fig. 24. GLC profile of metabolic end-products in ether extract from Bacteroides corrodens NCTC 10596 (333/54-55) representative of fermentation pattern II. Letters on peaks refer to retention times as shown in Table XIV.

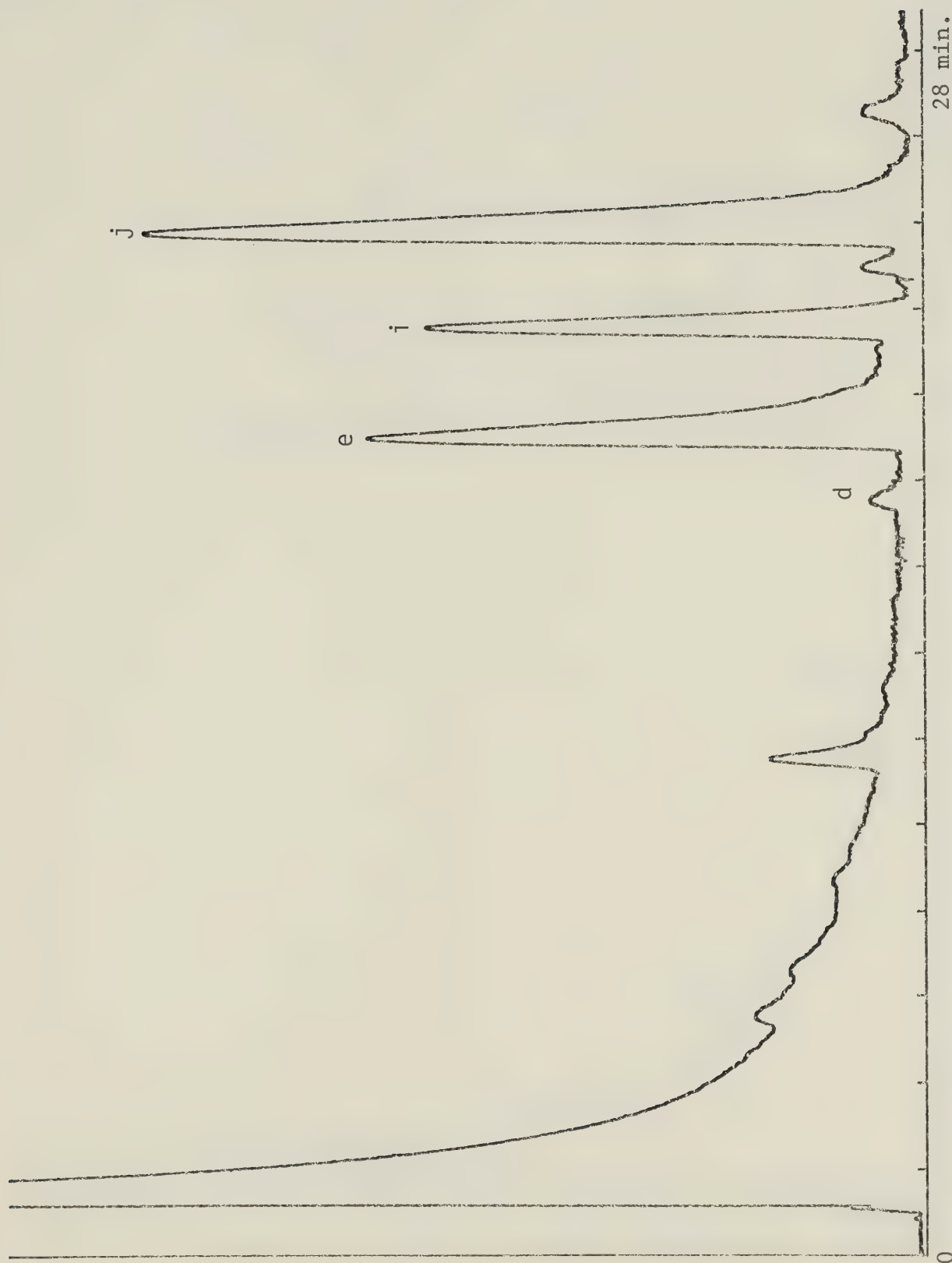


Fig. 25. GLC profile of metabolic end-products in ether extract from Bacteroides melaninogenicus representative of fermentation pattern III. Letters on peaks refer to retention times as shown in Table XIV.

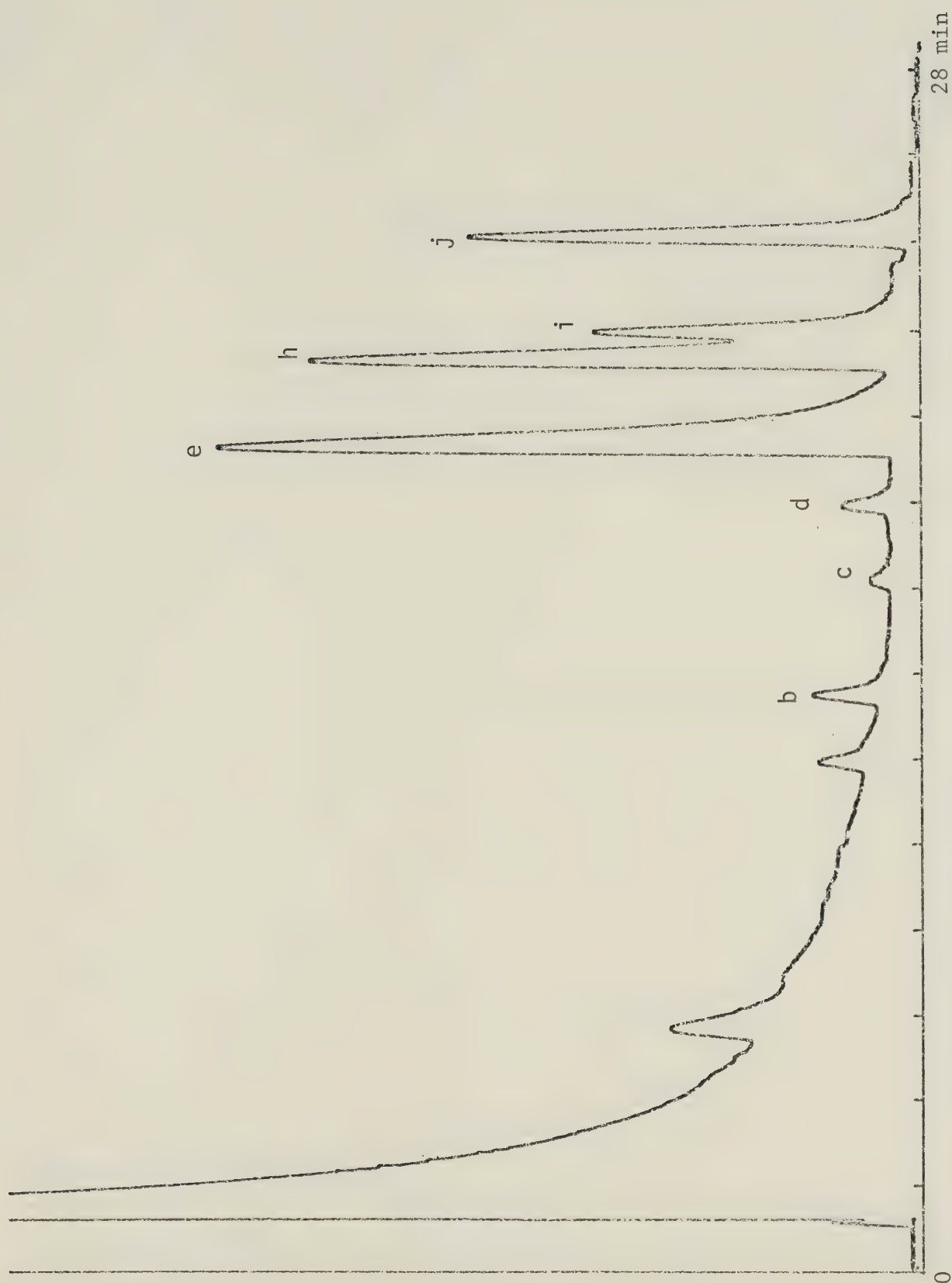
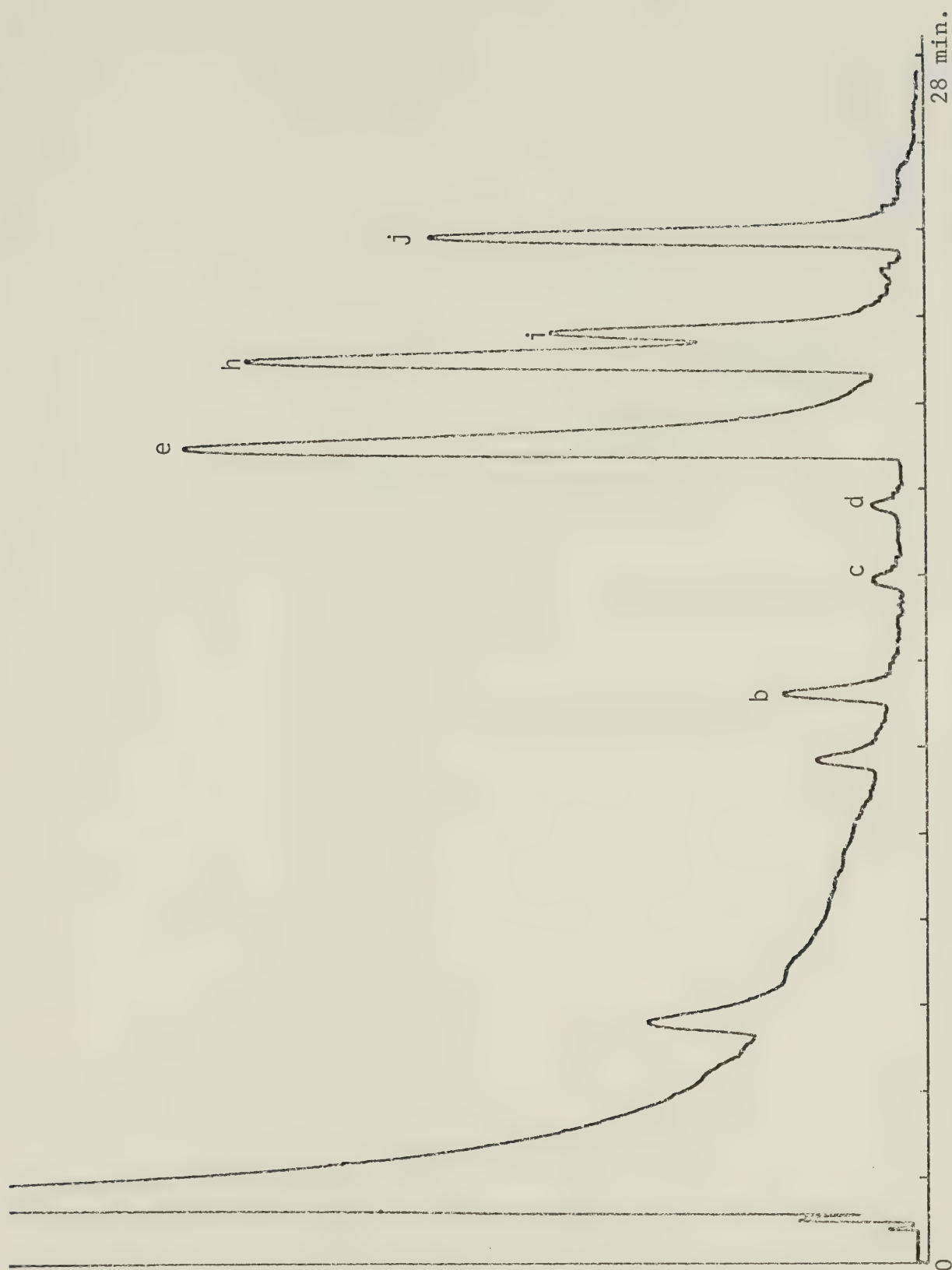


Fig. 26. GLC profile of metabolic end-products in ether extract from *Bacteroides fragilis* NCTC 9343 representative of fermentation pattern IV. Letters on peaks refer to retention times as shown in Table XIV.



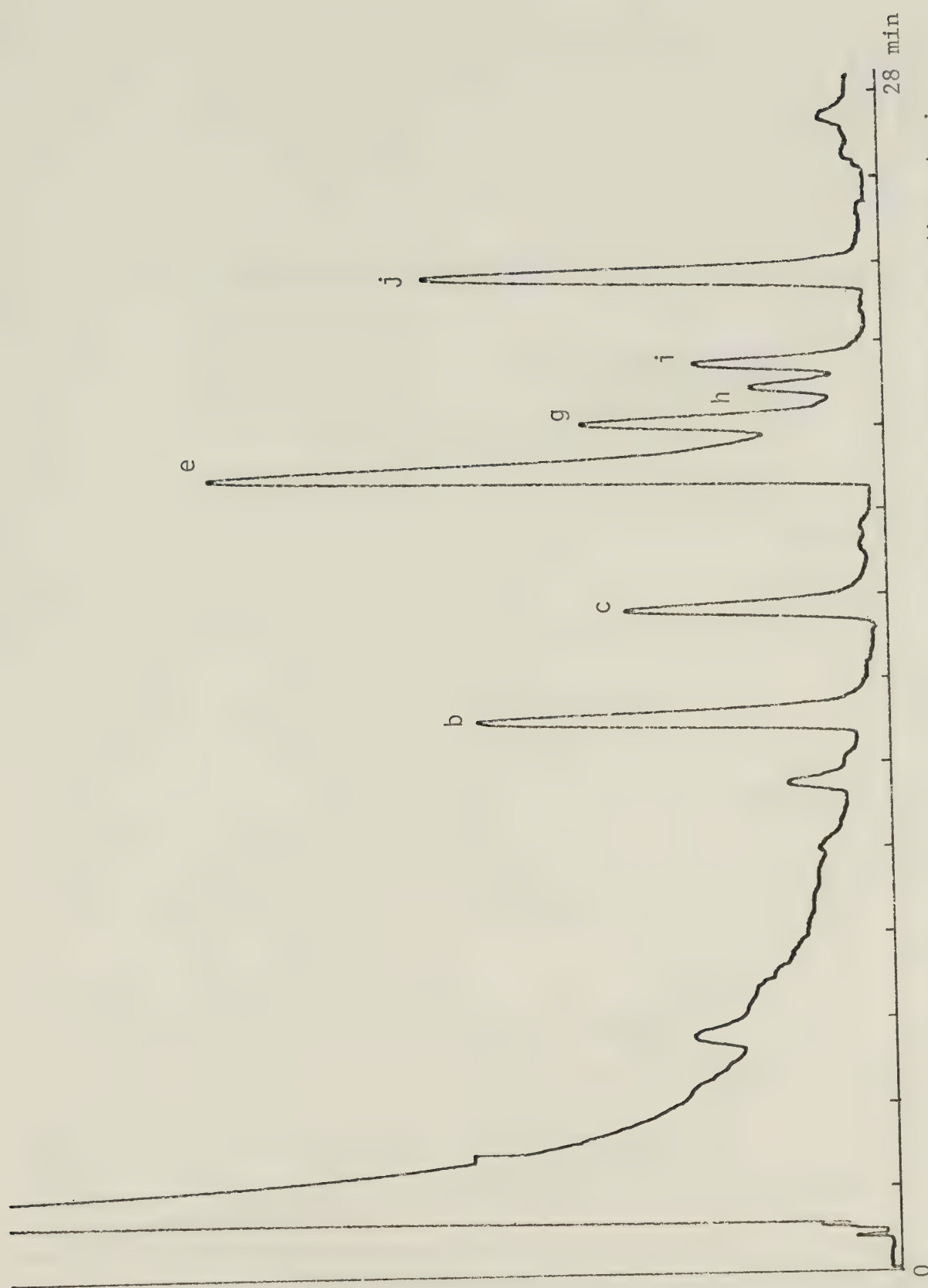


Fig. 28. GLC profile of metabolic end-products in ether extract from corroding strain 4482 representative of fermentation pattern V. Letters on peaks refer to retention times as shown in Table XIV.

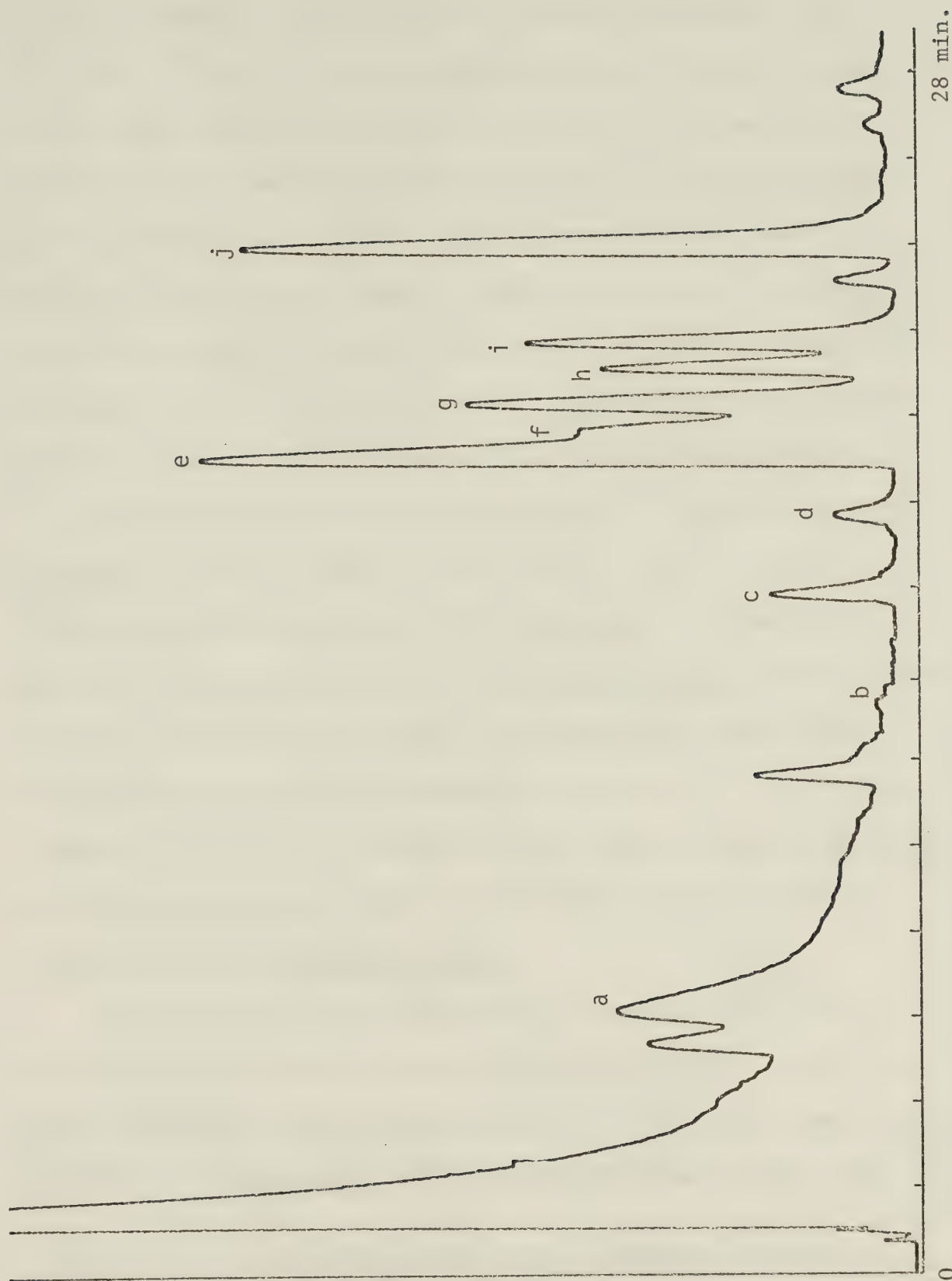


Fig. 29. GLC profile of metabolic end-products in ether extract from corroding strain 3936 representative of fermentation pattern V. Letters on peaks refer to retention times as shown in Table XIV.

strains resembled each other in that peaks designated as "e", "g", "h", "i" and "j" on the chromatograms are present in apparently equal proportions in both profiles. An incompletely resolved peak appearing as a shoulder on peak "e" and designated as "f" was detected in 3936 as well as an additional peak "a" which was not detected in any of the other strains of corroding bacilli described in previous fermentation patterns. Strain 4482 differed from 3936 and all the other strains of corroding bacilli in that peaks "b" and "c" also represented major end-products.

Fermentation pattern VI contained all of the strains of the Pasteurella genus included in this study. Fig. 30 is representative of the various serotypes of P. multocida. Fermentation profiles of P. haemolytica and P. pseudotuberculosis are illustrated by Figs. 31 and 32 respectively. Two detectable end-products characterized this group, the major end-product "e" being a major component in all of the strains in this study, "a" being shared only by the corroding strain 3936. End-product "a" appeared to be increased in P. pseudotuberculosis.

Fermentation pattern VII greatly resembled that of the facultatively anaerobic strains of corroding bacilli in that the major end-product detected was the same. This pattern was characteristic of H. aphrophilus NCTC 5886 and is shown in Fig. 33.

Evaluation of fermentation patterns as a taxonomic criterion

If the major end-products only were to be considered, fermentation patterns II, VI and VII could be grouped together, but this

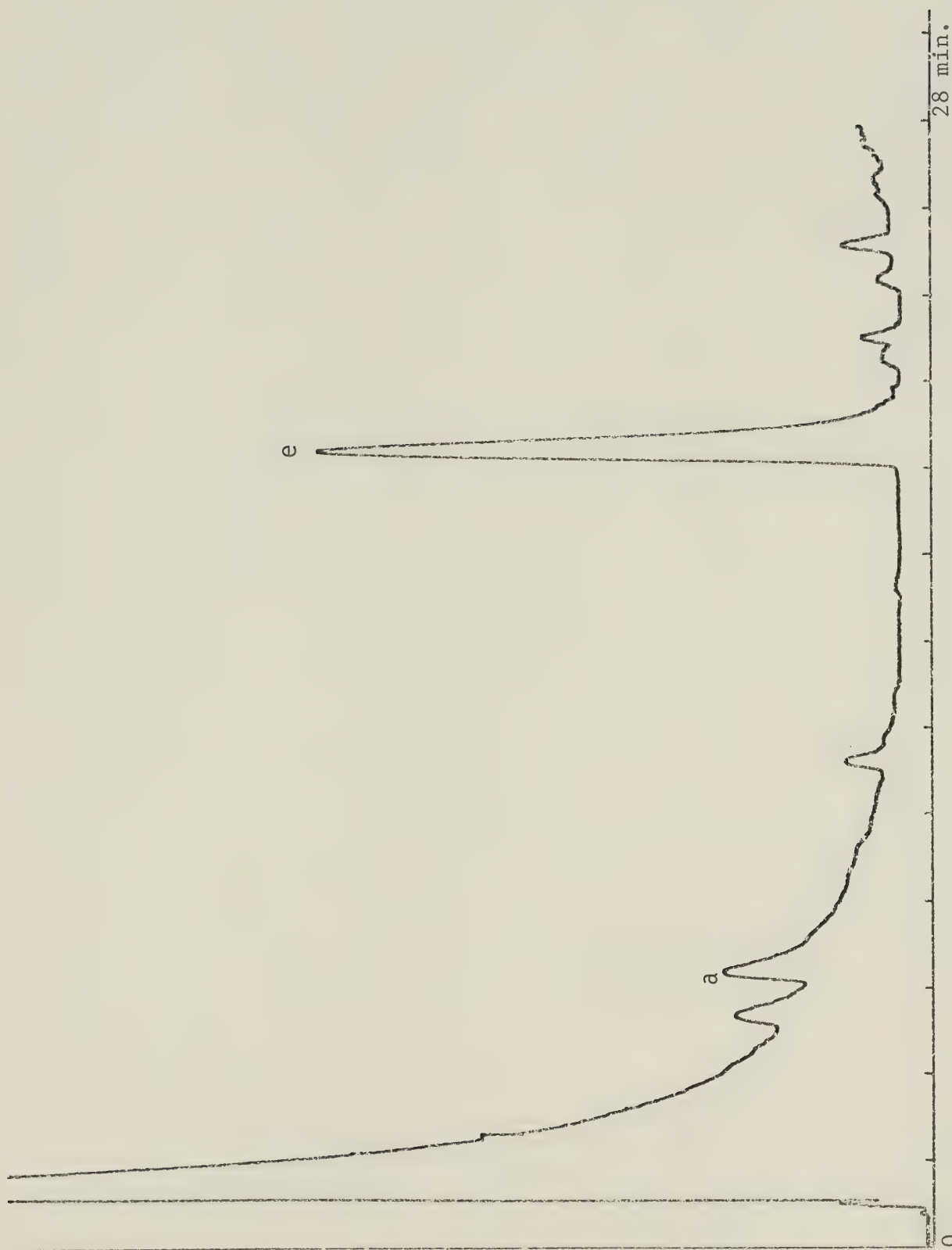


Fig. 30. GLC profile of metabolic end-products in ether extract from Pasteurella multocida C representative of fermentation pattern VI. Letters on peaks refer to retention times as shown in Table XIV.

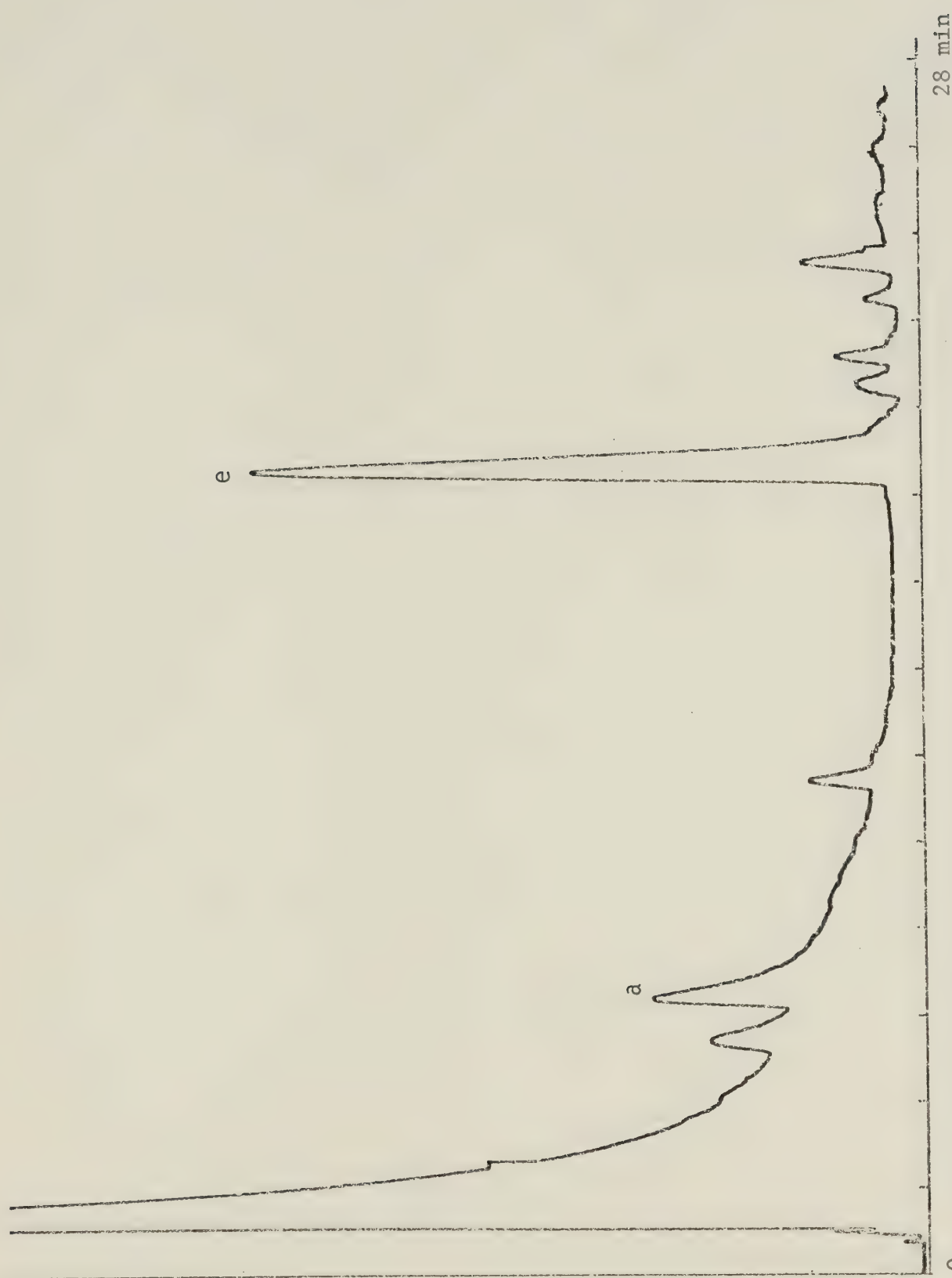


Fig. 31. GLC profile of metabolic end-products in ether extract from *Pasteurella haemolytica* representative of fermentation pattern VI. Letters on peaks refer to retention times as shown in Table XIV.

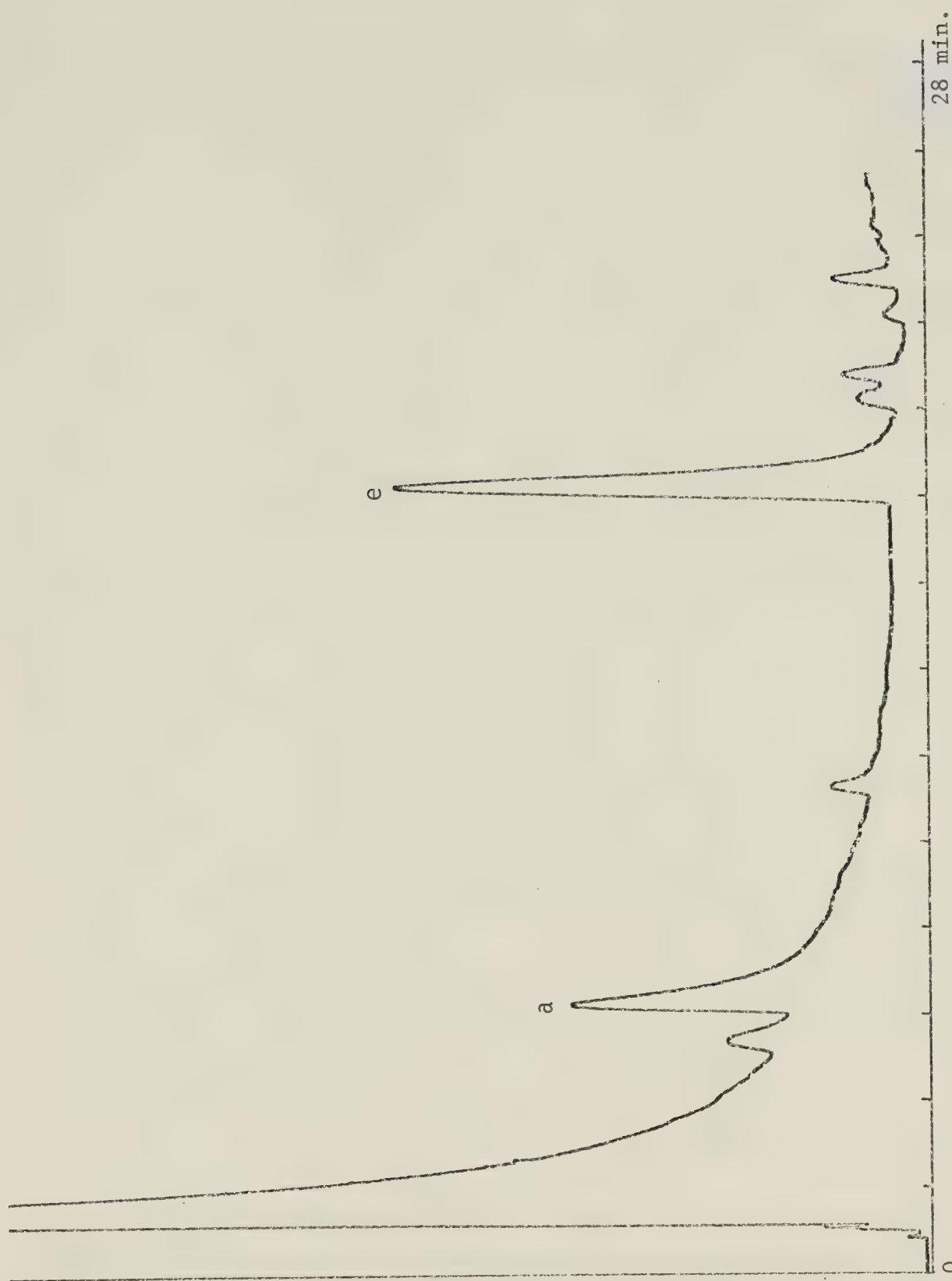


Fig. 32. GLC profile of metabolic end-products in ether extract from Pasteurella pseudotuberculosis representative of fermentation pattern VI. Letters on peaks refer to retention times as shown in Table XIV.

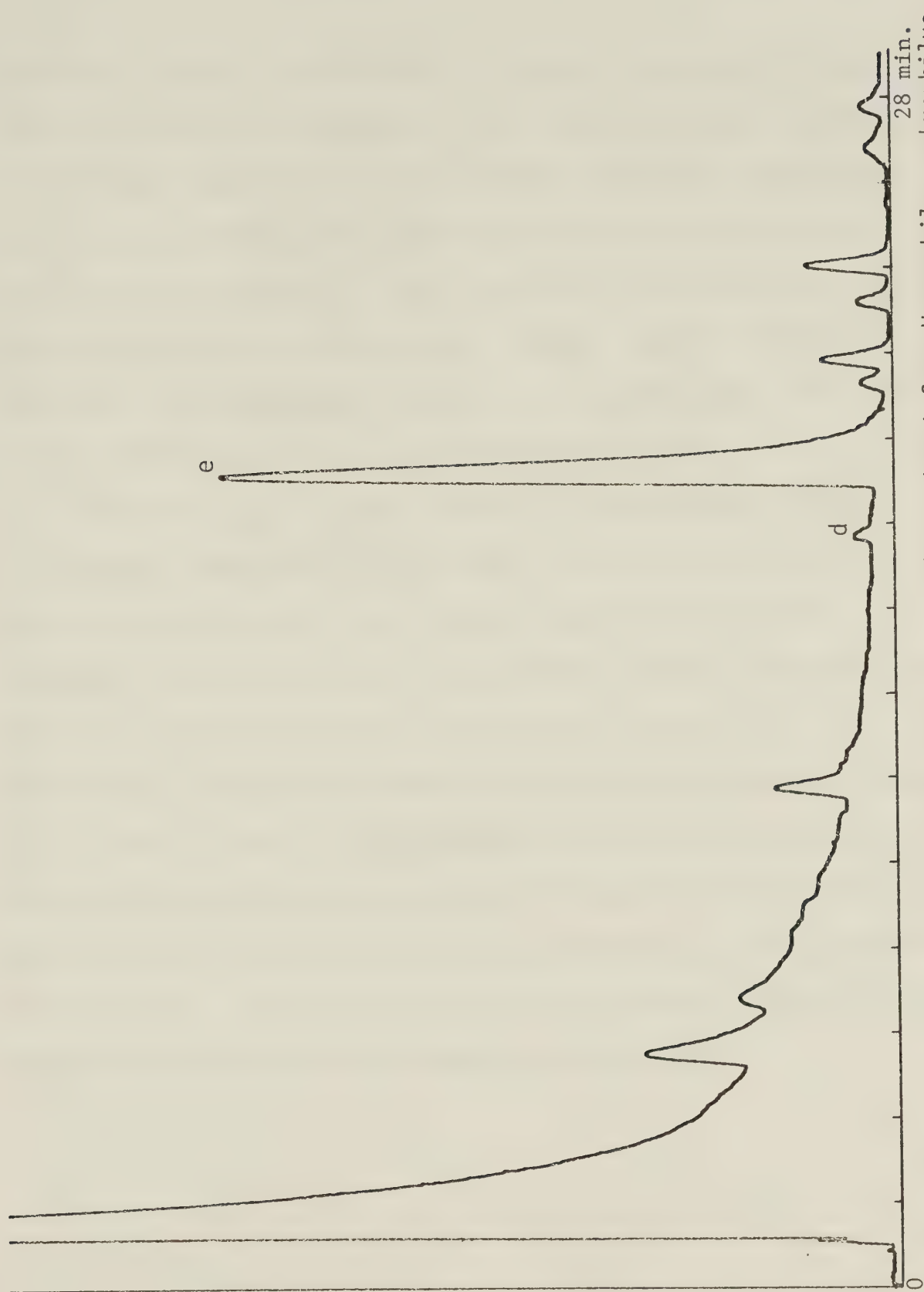


Fig. 33. GLC profile of metabolic end-products in ether extract from *Haemophilus aphrophilus* NCTC 5886 representative of fermentation pattern VII. Letters on peaks refer to retention times as shown in Table XIV.

would be illogical from the point of view of the varying cultural and biochemical characteristics of the organisms being considered. On the other hand, to distinguish between two groups of bacteria on the basis of metabolites present in trace amounts only could lead to false conclusions, since the detection of small peaks in a GLC profile is determined by several factors such as sensitivity settings of the instrument, sample size, concentration of the ether extract, efficiency of the extraction procedure, etc.

From this study it would appear that fermentation patterns by themselves would be of little value as a taxonomic criterion, but in conjunction with other criteria such as morphological, cultural, biochemical, and more specifically chemical characteristics, they would be of some value. For example, in part II of this dissertation, the microorganisms represented by fermentation patterns III, IV and V, namely, B. melaninogenicus, B. fragilis, B. oralis, 4482 and 3936 are grouped together on the basis of their cellular fatty acid composition but the group is described as being somewhat heterogeneous. The various fermentation patterns produced by these organisms illustrate the heterogeneity of this group.

IV

DISCUSSION

DISCUSSION

The genus Bacteroides as presently defined has become a repository of numerous strains of poorly characterized Gram-negative anaerobic bacilli. It may reasonably be said that too much attention has been paid to giving names to organisms, and not enough to gaining a meaningful understanding of the constitution and activities of the so-called 'species' included in the genus. In recent years, there has been a growing awareness of this among bacteriologists, and the need for reassessment of the anaerobic Gram-negative bacilli, as well as of certain micro-aerophilic organisms and facultative strains is widely recognized. Review of the literature shows that there has often been lack of reasonable standardization of methods for the study of these organisms and that, particularly with some strains, commonly used sets of conventional bacteriological tests may fail to give sufficient information to permit a meaningful classification.

The whole field of microbiological taxonomy and nomenclature is undergoing intensive review, and it is likely that important breaks will be made with long-established procedures, as recently suggested by Cowan (15). There is no doubt that the availability of computers will lead to their increased use in the handling of available data in taxonomy, and that there is a need for the collection of reliable information by well standardized methods to form the basis for a later more extensive analysis of organisms

of the type included in this study. The collection of reliable information facilitates classification, and at the same time provides a deeper understanding of the constitution and activities of the organisms, which is more important for microbiology than the precise nature of the code chosen for labelling strains. We should work towards a situation in which there is sufficient information available to permit reasonably accurate identification of strains of bacteria, so that organisms which have widely different genotypes are not masquerading under the same names in different laboratories. Because of genetic variation, it is to be expected that spectra of characteristics will be found in any group of closely related organisms, and the concept of a rigidly defined species will probably give way to delineation of population groups defined by some agreed measure of degree of interrelatedness between group members.

Rosebury (65) proposed a simplification of the classification of the organisms included in the Bacteroides and Sphaerophorus genera; he suggested that the organisms could be grouped into 10 species. Among these 10 representative species, B. fragilis would contain more than 20 types which are presently listed as separate species of the genus Bacteroides. The species B. funduliformis would contain all of the 18 types presently included in the genus Sphaerophorus as distinct species; B. melaninogenicus, the assacharolytic species and the motile species would be grouped separately.

In the present study, selected strains of anaerobic and facultatively anaerobic Gram-negative bacteria, which had been extensively studied by conventional methods in this laboratory, have been further analyzed by investigation of their fatty acid composition and metabolic end-products by means of gas-liquid chromatography. Particular attention has been paid to anaerobic and facultative 'agar-corroding' bacilli, because it is likely that different organisms which bear a superficial resemblance to each other have been confused in the literature, and all included under the name Bacteroides corrodens (26). Conventional testing, by means of carefully selected test series, had shown that there were three clearly distinguishable types (61), and on the basis of immunological tests, heterogeneity within two of the groups was demonstrated (26). It was therefore of interest to see to what extent fatty acid composition and fermentation patterns would permit the major groups and strains to be distinguished, and to assess the reproducibility of the tests and sensitivity to change of growth conditions, as variation in fatty acid profiles due to nutritional and/or environmental factors have been reported (17, 42, 70).

Several reports describing 'corroding bacilli' on the basis of cultural characteristics and different sets of conventional tests have been published (Eiken, 1958; Khairat, 1967, Henriksen, 1969). The strains studied by Khairat are no longer available, but appear to have been closely similar to our strains which are

designated EDMH-1, 4053, 4282 and B-912, although lack of precision in description of the methods used by Khairat makes exact comparison impossible. These strains are representative of those being named Bacteroides corrodens by North American microbiologists at the present time. Most available strains corresponding to the descriptions given by Eiken and Henriksen are facultative anaerobes, but Henriksen has designated the facultative strain 333/54-55 as the type strain of B. corrodens (24), as that name was first applied to these organisms by Eiken. This strain is in the National Collection of Cultures (Colindale, England) as NCTC10596. Work in our laboratory has shown that strains of this type usually require a rather high haemin concentration (50 $\mu\text{g/ml}$ or more) in the medium for aerobic growth, although non-haemin-requiring variants are encountered. Even with high haemin concentrations (50 $\mu\text{g/ml}$) the organisms remain catalase-negative (26).

Strains 4482 and 3936 are anaerobic, and while superficially similar to the other 'corroding bacilli', were distinguishable as a distinct type on the basis of biochemical (61) and immunological tests (Jackson, F. L. and Wong, P. C. unpublished). Commonly used tests give a pattern of reaction sufficiently similar to the other anaerobes to give rise to risk of confusion, although suitably chosen tests allow clear separation of the groups.

The results of the present study demonstrate the feasibility of distinguishing between the main groups of corroding bacilli by

GLC analysis of the methyl esters of their fatty acids and by GLC analysis of metabolic end-products liberated into the medium by the organisms. Visual comparisons of cellular fatty acid profiles and fermentation patterns allowed rapid differentiation of the facultative strains from the anaerobic strains. The latter were further subdivided into two groups (B-912, EDMH-1, 4053, 4282 and 3936, 4482).

These three groups of corroding bacilli each possessed characteristic fatty acid profiles and fermentation patterns which permitted them to be differentiated from each other by the presence or absence of certain peaks and by the relative proportions of the peaks present. All the strains within a group gave similar profiles. For further differentiation within a group, smaller but characteristic differences in some of the major fatty acid peaks could be used provided they were greater than the experimental variation, which ranged from 0% to 4% in the majority of the cases. Variation as great as 13.6% was however encountered in the unidentified fatty acid designated as K (ECL= 14.72) between the two replicates of Bacteroides fragilis. Differentiation within a group by comparisons of fermentation patterns was possible only with the anaerobic corroding strains 3936 and 4482.

On the basis of fatty acid spectra and metabolic end-products B. fragilis and B. oralis were indistinguishable but conventional tests have shown small biochemical differences between these strains.

De La Cruz and Cuadra (16), in an attempted classification of Bacteroides on the basis of antigenic characteristics have not observed any cross-agglutination between these two species.

There has been a tendency for bacteriologists to regard B. oralis as a biotype of B. fragilis rather than as a distinct species (66). The G+C contents of the strains used in this study were not significantly different, the values for B. oralis and B. fragilis being 43% and 42.2% respectively (61). It is apparent that the GLC conditions used in this investigation failed to distinguish between these two strains, which were separable on the basis of other tests. The 13.6% variation in a single peak, described earlier, was found in K (ECL =14.72) acid from B. fragilis. The independent position of B. melaninogenicus suggested by Rosebery (65) is evident from both the distribution of its fatty acids and its characteristic pattern of metabolic end-products. This has also been confirmed by Barnes (4) in a numerical analysis of Bacteroidaceae.

The fatty acid composition of the corroding strains 3936 and 4482 was shown to resemble that of the Bacteroides species included in this study. The major characteristic fatty acid, K (ECL =14.72), which varied from 62.9% to 70% in the Bacteroides strains investigated, represented 90.2% and 93.8% of the total fatty acids of 4482 and 3936 respectively. The increased proportion of this fatty acid in these corroding strains was much greater than that due to

experimental variation, hence this may indicate that these two organisms are distinct biotypes. Their fatty acid distributions were virtually identical, but differences appeared in the end-product chromatograms. In a series of conventional tests (61) it was found that 3936 hydrolyzed gelatin, whereas 4482 did not. There was some evidence that 4482 had weaker ornithine decarboxylase activity than 3936. These were the only differences in a set of 29 characteristics including DNA G+C content. These two closely related strains might therefore be regarded as biotypes of a species which can be distinguished from each other by end-product GLC analysis.

The facultative strains of corroding bacilli contained in Group I and the anaerobic strains comprising Group II were shown to be distinct from each other and from the representative accepted species of the genus Bacteroides included in this study both by their fatty acid composition and metabolic end-products. The grouping made on the basis of these two criteria correlate with certain biochemical characteristics and with G+C content (61). The facultative strains were shown to produce arginine, lysine and ornithine decarboxylase, whereas the anaerobic strains do not produce these enzymes but do produce urease, which the facultative strains do not. DNA base ratios of the facultative strains varied from 56.9% to 57.9% G+C; the base ratios of the anaerobic strains varied from 28.0% to 29.7% G+C.

Variation in the fatty acid pattern of the facultative strains

permitted differentiation of 53P and its non-pitting variant 53W from the other strains investigated. These two strains exhibited an amount greater than that due to experimental variation in octadecenoic acid with the concomitant decrease in palmitic acid. The fatty acid distributions of the anaerobic strains were essentially the same, hence differentiation within this group was not possible.

Group I strains which include the type strain NCTC 10596 (333/54-55) are all facultatively anaerobic and by definition, although some strains of B. fragilis can tolerate up to 8% oxygen (41), facultative strains are not at present admitted as Bacteroides species. Henriksen (23) suggests that the species designated B. corrodens does not fit into the genus Bacteroides but should be reclassified, and this opinion has been supported by Jackson et al. (26). Its resemblance to Moraxella kingii is pointed out. However, the G+C content of M. kingii is reported as 44.5% to 49% (quoted in 26).

Reclassification of the facultative strains and the anaerobic strains of Group II into another genus, or the creation of two new genera to accommodate them should not be undertaken until more Bacteroides and other related strains are investigated under the same cultural and experimental conditions. Members of the Bacteroides genus investigated were shown to be somewhat varied in their fatty acid composition, metabolic end-products, and biochemical characteristics. Heterogeneity in Bacteroides strains was

reported by Ifkovits and Ragheb (25) in a study of the fatty acids of rumen bacteria. Included in their investigation were three species of Bacteroides namely B. amylophilus, three strains of B. succinogenes and B. symbiosus. The fatty acid composition varied considerably from species to species. B. succinogenes was found to contain large proportions of $C_{13:0}$ and $C_{15:0}$ acids. B. amylophilus contained high concentrations of $C_{14:0}$, $C_{16:0}$ and $C_{18:1}$; B. symbiosus also contained large proportions of the fatty acids found in B. amylophilus in addition to $C_{17:0}$ and $i-C_{17:0}$. They found little relationship between cellular fatty acid composition of rumen bacteria except at the species level.

The fatty acid compositions of the anaerobic corroding strains investigated were shown to resemble qualitatively that of B. amylophilus as previously described. However, the complex medium used by Ifkovits and Ragheb (25) was supplemented with isovaleric, valeric, isobutyric and 2-methyl butyric acids. To what extent this would influence the fatty acid composition is difficult to assess until a comparison is made between this medium and the modified medium of Wahren and Holme used in this investigation.

The Pasteurella species which were included in this study for purposes of comparison were clearly distinguished from the Bacteroides species and the Group II anaerobic bacilli on the basis of their fatty acid composition. They shared one feature in common with Group I facultatively anaerobic corroding bacilli, in that palmitic acid was a major component of both groups. However, this

overlapping similarity is to be expected since palmitic acid is known to be the most common fatty acid in bacteria (53). The overall distribution of fatty acids between the Pasteurella species and the facultatively anaerobic corroding bacilli did show more differences than similarities.

Talbot and Sneath (67), in a numerical analysis of 59 strains of P. septica (multocida) from different origins, have shown them to be closely related to each other but distantly related to P. pestis and P. pseudotuberculosis, which are closely related to each other. In hybridization experiments (62) with P. tularensis, P. novicida, P. pestis, P. pseudotuberculosis and P. multocida, a distinct relationship was shown between the DNA of P. tularensis and P. novicida, and between P. pestis and P. pseudotuberculosis, thus indicating their genetic similarity. P. multocida was found to be unlike the others. In the present study the fatty acid composition of P. pseudotuberculosis was shown to be different from that of P. multocida strains by the presence of an unidentified fatty acid, T (ECL =16.83) and a lower concentration of palmitoleic acid. The fatty acid composition of P. pestis was not investigated, but a publication by Asselineau (3) on the fatty acids of this organism shows fairly good correlation with the results obtained for P. pseudotuberculosis in spite of the different experimental and cultural conditions. Asselineau reported the concentration of the following major fatty acids by plus signs: myristic acid, (+); palmitic, (+++); an unidentified C₁₇

cyclopropane acid, (++++); octadecenoic acid, (++) and an unidentified C₁₉ branched chain acid, (++)). The fatty acid pattern of our strain of P. pseudotuberculosis was: myristic acid, 2.4%; palmitic acid, 41.8%; and octadecenoic acid, 9.6%. Additionally, palmitoleic acid was found in a concentration of 28.8%. The unidentified fatty acid T (representing 14.4%), because of its position on the chromatogram and its ECL value of 16.83, could be a 17-carbon fatty acid. Inclusion of P. pseudotuberculosis in the genus Yersinia along with P. pestis as proposed by van Loghem (71) seems reasonable on the basis of these findings.

No qualitative differences in the fatty acid composition among the different serotypes of P. multocida were noted. The minor quantitative differences noted are probably not significant enough to distinguish among serotypes on the basis of their fatty acid composition alone.

The similarity between the fatty acid compositions of P. haemolytica and the P. multocida strains did not permit them to be distinguished from each other, but these two species were shown by Goodman to be serologically different (20).

On the basis of fatty acid composition, H. aphrophilus NCTC 5886 appears to be closely similar to the Pasteurella species, and this is in keeping with its family affiliation. This organism has certain superficial similarities to the facultative corroding bacilli, but the DNA G+C contents are significantly dissimilar (61). White and Cox (71), in a study of the fatty acids of H. para-

influenzae reported that palmitic, palmitoleic, oleic and vaccenic acids comprised 72% of the total fatty acids. In our strain of H. aphrophilus NCTC 5886, palmitic, palmitoleic and octadecenoic acids constituted 73.3% of the total fatty acids. It was not possible to include H. parainfluenzae or H. influenzae in this study because they did not grow on the medium used.

The purpose of this study was to compare the fatty acid composition and fermentation patterns of various bacteria. Variation in fatty acid profiles due to nutritional and/or environmental factors have often been observed, hence great care was taken to exclude this possibility. So far as was possible, cultures under comparison were grown under identical experimental conditions. Under these conditions the reproducibility was fairly good, as shown in Table V. A few experiments were carried out to see how nutritional and environmental factors might affect the groupings made in this study. The limited comparison of these factors indicated no problems in this respect.

Ideally, a completely defined medium should be used when comparing the fatty acid composition of various bacterial strains. Satisfactory defined media for the organisms used are not at present available. It is likely, however, that the use of defined media together with rigorous standardization of inoculum and of other growth conditions would lead to more exactly reproducible results, and so facilitate comparisons.

SUMMARY AND CONCLUSIONS

SUMMARY

Gram-negative, non-spore-forming, anaerobic and facultatively anaerobic bacilli tentatively designated Bacteroides corrodens have been compared by investigation of cellular fatty acid composition and metabolic end-products. They have also been compared with representative species of the genus Bacteroides. Members of the genera Pasteurella and Haemophilus were included as examples of genotypically different Gram-negative bacilli.

The fatty acids extracted from the cells were converted to methyl esters for GLC analysis. The metabolic end-products were extracted from the acidified culture supernatants with ether and chromatographed directly. Tentative identification of the fatty acids was performed by use of fatty acid methyl ester standards, chromatography on polar and non-polar columns and chromatography of bromo derivatives. Positive identification of some of these acids was established by combined gas chromatography-mass spectrometry. Equivalent chain length was calculated for all unidentified fatty acids.

Visual comparison of gas chromatographic methyl ester profiles of fatty acids extracted from the 26 cultures included in this study permitted separation into four distinct groups. The strains were grouped by observing the presence or absence, and relative sizes, of major peaks. Quantitative comparison of the percentages of the principal fatty acids supported the grouping

made by visual observations.

Organisms in Group I were characterized by large percentages of palmitic and octadecenoic acids with smaller percentages of palmitoleic acid and small amounts of lauric, myristic, and stearic acids. This fatty acid spectrum was characteristic of the facultatively anaerobic strains of corroding bacilli:

Bacteroides corrodens NCTC 10596 (333/54-55), B. corrodens NCTC A40/68 and the similar strains tentatively designated B. corrodens, namely B-916, S-209, 53P and 53W.

Group II organisms contained a large percentage of octadecenoic acid and smaller amounts of palmitic, lauric, myristic and stearic acids. Four of the anaerobic strains of corroding bacilli also tentatively designated B. corrodens were grouped together on the basis of this characteristic fatty acid profile. These were: B-912, EDMH-1, 4053 and 4282 (Lane).

Group III was characterized by an unidentified acid with an ECL of 14.72 which represented as much as 93% of the total fatty acids. This fatty acid was found in three accepted members of the genus Bacteroides, namely B. fragilis, B. oralis and B. melaninogenicus and also in two anaerobic corroding bacilli tentatively designated B. corrodens: 3936 and 4482. B. melaninogenicus was shown to differ from the other species of this group on the basis of an unidentified fatty acid. This group was not as homogeneous in its fatty acid distribution as the other groups.

Group IV contained all of the Pasteurella species included

in this study: P. multocida A, C, D, NT, P. haemolytica and P. pseudotuberculosis. This genus was characterized by large percentages of palmitoleic and palmitic acids in almost equal proportions, and a smaller percentage of myristic acid. Variation in fatty acid distribution among the various serotypes of P. multocida was not great enough to use as a basis for their differentiation. P. pseudotuberculosis was shown to vary from the other species by an unidentified fatty acid (ECL=16.83) which was unique to this species of Pasteurella examined. Haemophilus aphrophilus, once suggested as being related to the corroding strains, was more closely similar to this group.

The fatty acid patterns sometimes permit recognition of small but significant differences between closely related organisms, but in other instances conventional bacteriological tests may reveal differences which are not reflected in the fatty acid pattern.

Investigation of the metabolic end-products produced by these organisms revealed seven distinct fermentation patterns. Identification of the metabolites was not attempted. Those organisms placed in Groups I and IV on the basis of their cellular fatty acids each had their characteristic fermentation pattern, provided the major end-products and those present in trace amounts were taken into consideration. Haemophilus aphrophilus had its characteristic fermentation pattern, as did the anaerobic corroding bacilli of Group II. However, if only the major end-products were considered, Group I, IV and H. aphrophilus could be grouped

together. Organisms in Group III exhibited three distinct fermentation patterns. Fermentation patterns by themselves would be of little value as a taxonomic criterion but would be a valuable aid in conjunction with other criteria. This was evident in the demonstration of heterogeneity of the organisms placed in Group III on the basis of their cellular fatty acid composition.

It was evident from this study that fatty acid composition as a taxonomic criterion can be useful because a spectrum of characteristics can be determined rapidly and efficiently by gas-liquid chromatography; however, it is necessary to use characteristics determined by a number of different techniques as a basis for taxonomic studies.

CONCLUSIONS

The consideration of chemical composition and more specifically fatty acid composition as a taxonomic criterion has been discussed in several publications. The sensitivity and rapidity with which fatty acids can be studied with the help of gas-liquid chromatography makes this approach attractive for the classification of microorganisms even though GLC alone cannot always be used for positive identification of fatty acids. However, tentative identification of separated components can be made or the chromatograms can be used for direct visual comparisons. The results in this investigation were not affected by change of medium and growth conditions. However, effects of changes in nutritional and environmental conditions must be kept in mind when determining fatty acid composition of bacteria.

In this study differentiation at the family level was not possible owing to there being too few families represented. The detailed distribution of fatty acids did permit differentiation at the genus level. Although there were overlapping similarities between the genus Pasteurella and Group I facultatively anaerobic corroding bacilli, the pattern remained characteristic for the genus. Differentiation at the species level was possible in some cases, for example, Bacteroides melaninogenicus was distinguished from B. fragilis and B. oralis; Pasteurella pseudotuberculosis was distinguished from P. haemolytica and P. multocida. On the

other hand, the closely related B. fragilis and B. oralis were not differentiated from each other, neither were P. haemolytica and P. multocida. However, knowledge of the cellular fatty acid distribution in these species may be of value in augmenting classification by other recognized procedures.

End-product analysis by the method used was of little value as a taxonomic criterion by itself, but in combination with other criteria it could be useful. In this investigation, fermentation patterns were of value in indicating the differences between members of the genus Bacteroides and the unidentified isolates tentatively placed in this genus.

On the basis of these two criteria, the problem of clarification of the taxonomic status of Gram-negative, non-spore-forming, agar-pitting, anaerobic and facultatively anaerobic bacilli tentatively designated B. corrodens was resolved in the following manner:

The anaerobic strains of corroding bacilli B-912, EDMH-1, 4053 and 4282 (Lane) and the facultative strains B. corrodens NCTC 10596 (333/54-55), B. corrodens A40/68, B-916, S-209, 53P and 53W need to be reclassified. The fatty acid composition of the anaerobic strains bears no resemblance to that of the facultative strains. The anaerobic strains produced no end-products detectable by the methods used. The investigation of certain enzymatic activities may be of value in the characterization of these organisms since the facultative strains produce arginine, lysine and ornithine-

decarboxylases whereas the anaerobic strains do not produce these enzymes but do produce urease, which the facultative strains do not. Characterization of Bacteroidaceae on the basis of dehydrogenase patterns by Keudell and Goldberg (32) proved useful in the clarification of the taxonomic relationships of this family.

The finding that these two groups had high proportions of normal, even-numbered, saturated and unsaturated fatty acids agreed closely with the general features of Gram-negative bacteria as indicated by Kates (31).

The necessity for further investigation of these organisms is evident from the current proposed classification. This illustrates that many criteria, morphological, biochemical, serological, toxicological, along with analysis of chemical components such as lipids and DNA base ratios may be utilized to attain a satisfactory classification. Amino acid sequences of enzymes, and DNA homology studies may later help to clarify the interrelationships of micro-organisms.

VI

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VII

APPENDICES

APPENDIX A

Nomenclature of fatty acids discussed in this study

Systematic name	Common name	Chemical formula
Normal saturated fatty acids		
ethanoic	acetic	$C_2H_4O_2$
butanoic	butyric	$C_4H_8O_2$
pentanoic	valeric	$C_5H_{10}O_2$
hexanoic	caproic	$C_6H_{12}O_2$
heptanoic	heptylic*	$C_7H_{14}O_2$
octanoic	caprylic	$C_8H_{16}O_2$
nonanoic	pelargonic	$C_9H_{18}O_2$
decanoic	capric	$C_{10}H_{20}O_2$
undecanoic	undecylic	$C_{11}H_{22}O_2$
dodecanoic	lauric	$C_{12}H_{24}O_2$
tetradecanoic	myristic	$C_{14}H_{28}O_2$
pentadecanoic	pentadecylic	$C_{15}H_{30}O_2$
hexadecanoic	palmitic	$C_{16}H_{32}O_2$
heptadecanoic	margaric	$C_{17}H_{34}O_2$
octadecanoic	stearic	$C_{18}H_{36}O_2$
eicosanoic	arachidic	$C_{20}H_{40}O_2$
docosanoic	behenic	$C_{22}H_{44}O_2$
Unsaturated fatty acids		
undecenoic	undecylenic	$C_{11}H_{20}O_2$
9-hexadecenoic	palmitoleic	$C_{16}H_{30}O_2$

APPENDIX A (Contd.)

6-octadecenoic	petroselinic	$C_{18}H_{34}O_2$
cis-9-octadecenoic	eleic	$C_{18}H_{34}O_2$
trans-9-octadecenoic	elaidic	$C_{18}H_{34}O_2$
trans-11-octadecenoic	vaccenic	$C_{18}H_{34}O_2$
cis-9, cis-12-octadecenoic	linoleic	$C_{18}H_{32}O_2$

Branched-chain fatty acids

2-methylpropionic	isobutyric	$C_4H_8O_2$
3-methylbutanoic	isovaleric	$C_5H_{10}O_2$

* also called enanthic acid.

APPENDIX B

Culture Medium

1. Wahren and Holme medium modified for fatty acid analysis.

A.	Yeast extract (Difco)	5 g
	Tryptone (Difco)	15 g
	Sodium chloride	2.5 g
	Sodium thoglycollate	0.5 g
	Potassium hydrogen phosphate (anhydrous)	2.5 g
	Bacto agar	15 g
	Distilled water	926 ml

Autoclaved at 121°C for 15 min

B.	20% glucose solution	50 ml
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Autoclaved at 110°C for 12 min

C.	5% sodium bicarbonate	20 ml
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Filter sterilized

D.	Haemin (Eastman Organic Chemicals) stock stock solution: 5 mg/ml in 0.5% $\text{Na}_2\text{CO}_3 \cdot 2\text{H}_2\text{O}$	
	Filter sterilized.	

E.	Cystine 0.05 g dissolved by boiling in 4 ml 0.25N HCl	
	Prepared prior to use.	

Solutions B, C and E added to A aseptically. Solution D added to give a final concentration of 5 µg/ml.

2. Wahren and Holme medium modified for end-product analysis.

Difco peptone (1%) was substituted for tryptone in solution A. Bacto agar was omitted.

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